

**THERAPEUTIC IMMUNOMODULATION OF ALLERGIC LUNG DISEASE USING
REGULATORY DENDRITIC CELLS IN A MOUSE MODEL OF ASTHMA.**

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By

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ABSTRACT

We report herein that IL-10-treated dendritic cells (DC) can be used effectively to reverse established severe asthma-like disease in a mouse model. Our lab had shown previously that allergen-presenting splenic CD8 α^+ DCs could \approx 50% reduce airway hyper responsiveness (AHR), eosinophilia, and Th2 responses in asthma-phenotype mice, but only marginally reduce IgE/IgG1 levels. We now show that bone marrow-derived DCs that have been differentiated in the presence of IL-10 (DC_{IL-10}) are effective in reversing the asthma phenotype. Co-culture of DC_{IL-10} with T memory (T_M) cells from asthma-phenotype mice was associated with lack of Th2 responses, and this was partially reversed by IL-2. Immunostimulatory DC activated these Th2 cells. *In vivo*, delivery of allergen-pulsed DC_{IL-10}, either into the airway or intraperitoneally abrogated AHR from weeks 3-10 post-treatment, and ameliorated lung eosinophilia and Th2 (IL-4, -5, -9, & -13, IgE) responses, as well as circulating allergen-specific IgE responses for at least 32 weeks following treatment. Repeated ^{OVA}DC_{IL-10} treatments kept AHR normalized for 8 weeks as well as Th2 responses significantly low. *In vivo*, delivery of anti-IL-10R, but not anti-TGF- β from day 12-21 after treatment had moderate effects on DC_{IL-10}-driven tolerance, but 1-methyl tryptophan (inhibitor of indoleamine-2,3-dioxygenase) treatment had significant effects on Th2 responses. The mechanisms mediating tolerance *in vivo* are likely complex, but we speculate that infectious tolerance sustains this regulatory activity during the 32-week period in which we have observed tolerance to be in place.

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DEDICATION

*I dedicate my thesis to my
Parents
Kusam and Vijay Kumar Nayyar
whose efforts and ever-willing support have made this dream come true.*

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LIST OF ABBREVIATIONS

Ab	antibody
AEC	alveolar epithelial cells
Ag	antigen
AHR	Airway hyperresponsiveness
ALD	allergic lung disease
Alum	aluminum hydroxide
AM	alveolar macrophage
APC	antigen presenting cell
ASM	airway smooth muscle
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BDCA	blood-derived cell antigen
BM-DC	bone marrow derived dendritic cells
CCR	chemokine receptor
CD	cluster of differentiation
CpG	cytosine-guanine dinucleotides
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DNA	deoxyribonucleic acid
EAR	early asthmatic reaction
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorter
Fc	crystallizable fragment of Ab
FEV ₁	forced expiratory volume in 1 sec
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/monocyte-colony stimulating factor
HLA-DR	human leukocyte antigen-DR

i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule-1
IDO	indoleamine 2, 3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.t.	intratracheal
KO	knockout
LAG-3	lymphocyte activation gene-3
LAR	late asthmatic reaction
LPS	lipopolysaccharide
LSM	lymphocyte separation medium
LT	leukotriene
M	muscarinic
Mab	monoclonal antibodies
MBP	major basic protein
Mch	methacholine
MCP	monocyte chemotactic protein
MDC	monocyte derived chemokine
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger RNA
NK	neurokinin
NO	nitric oxide
OVA	ovalbumin
PAF	platelet activating factor
PAR	proteinase activating receptor
PBMC	peripheral blood leukocyte
Pg	picogram

PG	prostaglandin
PSGL	P-selectin glycoprotein ligand
qRT-PCR	quantitative real-time polymerase chain reaction
r	recombinant
R	receptor
RANTES	regulated on activation normal T cell expressed and secreted (chemokine)
RNA	ribonucleic acid
s.c.	subcutaneous
SEM	standard error of the mean
SIT	specific immunotherapy
SP	substance P
TARC	thymus and activation regulated chemokine
TcR	T cell receptor
TGF-	transforming growth factor
TLR	toll-like receptors
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
TX	thromboxane
VCAM-1	vascular cell adhesion molecule-1
VIP	vasoactive intestinal peptide
VLA-1	very late activation-1
µg	microgram
µl	microlitre

1.0 INTRODUCTION

Despite significant pharmacological advances in asthma therapy, the past two decades have seen alarming increases in the prevalence of this disease world-wide (American Lung association Epidemiology and statistics unit, March 2003). Allergic asthma is characterized by intermittent and reversible airway obstruction, airway hyperresponsiveness (AHR), and airway inflammation (Galli 1999), but these are driven by underlying Th2-type immune responses. Conventional treatments have not addressed the immunologic basis of this disease, but rather are primarily symptomatic in nature, targeting either the AHR (e.g., bronchodilators) or inflammatory responses (e.g., steroids) (Lin and Casale 2002). More refined approaches have included administering anti-IgE (Finn, Gross *et al.* 2003) or anti-cytokine, or anti-cytokine receptor antibody (Tomkinson, Duez *et al.* 2001; Foster, Hogan *et al.* 2002; Leckie 2003) or leukotriene antagonists (Ducharme, Hicks *et al.* 2002; Asakura, Ishii *et al.* 2004). Despite these efforts the incidence and severity of asthma continues to rise (Galli 1999). Co-administration with allergen of cytosine-guanidine motif-rich oligodeoxynucleotides reduces airway eosinophilia, Th2 cytokine induction, IgE production, and bronchial hyperreactivity in mouse models of (Lin and Casale 2002), but they do so by skewing of the allergic inflammatory responses to Th1-dominant mode, which itself carries significant health risks (i.e. allergen-driven hypersensitivity pneumonitis). Tolerance is strictly defined as the absence of an immune response but over the past few years, this concept has changed. Now tolerance is described as an active process which involves the same principles as self-tolerance i.e. deletion, anergy and

suppression. In principle, it would seem that immunologic tolerization could be a more appropriate means of reducing asthma pathology than would immune deviation¹.

Dendritic cells (DC) are initiators and modulators of immune responses. They are thought to be the most powerful of all antigen-presenting cells that can activate naïve T cells (Banchereau and Steinman 1998). The capacity to induce active immunity versus tolerance (via regulatory cells) is in large part controlled at the level of the dendritic cell. Immature or quiescent DCs can induce antigen-specific T cell tolerance, whether the DCs are differentiated *in vitro* (Jonuleit, Schmitt *et al.* 2000) or *in vivo* (Gordon, Li *et al.* 2005). But induction of tolerance by DCs does not necessarily depend exclusively on their not having been exposed to inflammatory environments, since LPS-activated CD4⁺/CD8⁻ splenic DCs can also potently induce tumor tolerance via up regulation of regulatory T cells (Zhang, Huang *et al.* 2005). The ability of tolerogenic DC to prevent experimental antigen sensitization have been documented in model systems ranging from cancer (Steinbrink, Jonuleit *et al.* 1999) to allergic diseases (Muller, Muller *et al.* 2002), but few had reported on the ability of such cells to reverse the course of a pre-existing immunologic sensitivity. We recently reported that allergen-pulsed, otherwise quiescent splenic CD8 α ⁺ DCs can dampen allergic pathology in a mouse model of allergic asthma, although the reversal of the asthma phenotype was not complete (Gordon, Li *et al.* 2005). We did document a role for both contact-dependent and independent mechanisms (e.g., IL-10, indoleamine-2,3-dioxygenase; IDO) in this process (Gordon, Li *et al.* 2005).

We wished to determine however whether we could increase the effectiveness of our

¹ “immune deviation” was coined by Asherson in 1965. Few years later, inverse relationship between humoral and cell-mediated responses and vice versa defined this phenomenon. In mid to late 1970’s, immune deviation mediated by active T cell suppression represented “immunological tolerance”. Although in broader sense, it means a modified response to an antigen but with the emergence of Th1/Th2 paradigm, the term has been used in a more refined manner i.e. skewing Th2 to Th1 response (also used similarly for this thesis). With the discovery of regulatory T cells definition of tolerance has changed over time from “non-reactivity against self” to an active physiological process of non-pathological state wherein regulatory responses dominate over pathological responses without the Th1/Th2 bias.

tolerization of the asthma-like phenotype by use of alternate dendritic cell-based strategies. Interleukin (IL)-10 is an anti-inflammatory cytokine known to have inhibitory effects on accessory functions of DCs (Muller, Muller *et al.* 2002), perhaps by inhibiting cellular maturation, such that IL-10-treated DCs express a tolerogenic phenotype (Steinbrink, Wolfl *et al.* 1997; Steinbrink, Graulich *et al.* 2002). IL-10-modulated DCs can inhibit proliferation of, and cytokine production by, differentiated T cells and thereby induce antigen-specific anergy, even after a single contact with T effector cells. Indeed, DCs recovered from IL-10 secreting melanoma tumors anergize anti-tumor responses, and this can be reversed by neutralizing IL-10 in this system and recapitulated *in vitro* by treating DCs with exogenous IL-10 (Steinbrink, Wolfl *et al.* 1997; Steinbrink, Jonuleit *et al.* 1999). Interestingly, it was recently reported that IL-10-differentiated, allergen-presenting DC are completely ineffective in reversing the asthma phenotype after i.v. delivery to mice (Bellinghausen, Sudowe *et al.* 2006), while TGF- β -differentiated bone marrow-derived DC also only modestly reduce the localization of allergen sensitivity to the lungs of systemically sensitized mice (Zhang-Hoover, Finn *et al.* 2005). The few studies so far have either been done when DC_{IL-10} therapy is given before or during the sensitization phase or before the aerosol challenge (Zhang-Hoover, Finn *et al.* 2005; Bellinghausen, Sudowe *et al.* 2006; Koya, Matsuda *et al.* 2007). Recently, it was reported that DC engineered to express IL-10 induce prolonged tolerance in asthma model (Henry, Desmet *et al.* 2008) but the work presented in this thesis predates the above mentioned papers.

Herein we explore the therapeutic usefulness of delivering tolerogenic DC to asthma-phenotype, or ‘asthmatic’, mice, using bone marrow cells that are differentiated *in vitro* in the presence of IL-10. In this study we generated tolerogenic DCs by culture of immature mouse bone marrow DCs in IL-10 (DC_{IL-10}) and show that after pulsing with allergen, these cells can

abrogate AHR in a mouse model of severe allergic lung disease (ALD), and markedly attenuate all other pathologic characteristics of the asthma-like phenotype in these animals. We show that these cells can provoke a prolonged and highly effective allergen-specific tolerization of the pulmonary and systemic allergic responses in the treated animals.

2.0 REVIEW OF LITERATURE

2.1 IMMUNOPATHOLOGY OF ASTHMA

2.1.1 Definition, epidemiology and possible etiology

Asthma is a chronic obstructive disease of the small airways of the lung and is characterized by varying degrees of intermittent and reversible airway obstruction and exaggerated airway responsiveness to a wide variety of physical and pharmacological agents or non-specific stimuli, in association with pathological inflammatory changes in the bronchial tree. All the above factors together with related chronic exposure to allergen leads to airway remodeling, including epithelial denudation, mucosal edema, mucus hypersecretion, sub-epithelial fibrosis, increased airway vascularization, and smooth muscle hyperplasia. This further limits airflow and is clinically manifested as paroxysms of dyspnea, wheezing, cough and chest tightness (Bousquet, Jeffery *et al.* 2000; Busse and Lemanske 2001).

Being a multifactor and complex disease, asthma involves interactions between, for example the respiratory, immune, circulatory and neuronal systems, and both genetic (familial tendency to become sensitized and produce IgE antibodies in response to allergens (Cookson 1999)) and environmental factors that contribute to its initiation and progression. The environmental factors include e.g. viruses (Lemanske 2004), allergens (Platts-Mills, Sporik *et al.* 1997), and occupational exposure (Chan-Yeung, Malo *et al.* 2003). The classification of asthma has been recently revised to include allergic asthma (atopic or extrinsic asthma), resulting from

immunological reactions to common environmental antigens, and non-allergic asthma (non-atopic or intrinsic asthma) caused by unknown antigens, possibly triggered by viral or autoantigens (Humbert, Menz *et al.* 1999; Johansson, Bieber *et al.* 2004). For the purpose of this thesis, we will for the most part address only allergic asthma, as treatment in our model targets the underlying immunoinflammatory pathology caused by Ovalbumin-induced allergic responses.

Asthma is now recognized as an epidemic, as it affects 2% of the world population and 8-10% of the population of US. In 2003, it was estimated that 20 million Americans had asthma, including 6.2 million children under age 18 and 1.2 million under age 5. Of these, 11 million Americans, including 4 million children under 18, had had an asthma attack that year (National centre for health statistics. Raw data from national health interview survey 2003). The annual direct health care as well as indirect (e.g. lost productivity) costs of asthma in the US total approximately 1.6 billion dollars (National heart 2004). According to Statistics Canada, 8.4% of the population (aged 12 and over) has been diagnosed with asthma (2000-2001) and asthma has been reported to affect at least 12% of Canadian children. Direct costs of asthma, which include medical/nursing care and medication, in Canada were estimated at \$600 million per year. In 1994, the cost of hospitalization alone for asthma was \$135 million. The total cost of asthma care in Canada was estimated between \$504 and \$648 million dollars annually (reviewed in Fact sheet N° 1, revised 2005, Asthma Society of Canada).

2.1.1.1 Overview of Pathophysiology of Asthma

The pathophysiology of asthma is complex, involving a wide variety of cells interacting in different ways, leading to events ranging from acute to chronic phases of inflammation. To

have a clear picture of the pathophysiology I have summarized the events that take place sequentially.

In the phase of sensitization (Figure 1.1a), on its first encounter with the host the allergen is taken up by immature DCs located under the airway epithelium. The antigen is directed to the endocytic pathway of APC where it is degraded and presented on the cell surface as MHC class II-peptide complexes. During this process DC undergo characteristic maturational changes and move under the influence of chemokines to the lymphoid organs, where they interact with and stimulate naïve T cells (Vermaelen, Carro-Muino *et al.* 2001; Vermaelen and Pauwels 2005). The naïve T cells then receive signals driving them towards Th2 response mode, depending on the hosts' genetic status, the nature of the allergen, the microenvironment within lung and the inherent tendency of lung DCs to induce allergic response (Stumbles, Thomas *et al.* 1998). In addition, extrinsic factors like viral infections and endotoxins have been reported to predispose to asthma (Schwarze and Gelfand 2002; Thorne, Kulhankova *et al.* 2005). In the presence of IL-4 secreted by T cells and CD40-CD40L interaction between T and B cells allergen-specific IgE is produced, and this binds FcεRI receptors on mast cells, basophils or APCs, increasing the efficacy of allergen responses on re-exposure. Finally, the sensitization phase involves the formation of a pool of allergen-specific memory CD4 T cells and B cells. Memory T cells can both home to peripheral lymphoid organs (central memory) or inflamed tissues (effector memory) and require low stimulation from DC to become activated (Medoff, Thomas *et al.* 2008).

The elicitation phase: This phase comprises an early (EAR; Figure 1.1b) and a late asthmatic reaction (LAR; Figure 1.1c). Re-exposure of a sensitized individual to specific allergen cross-links the cell-bound IgE on mast cells etc, and leads within minutes to the immediate

release of preformed mediators such as histamine and the synthesis of leukotrienes and prostaglandins. The actions of these mediators' accounts for the immediate symptoms (EAR), including mucus production, smooth muscle constriction, vasodilation, and increased vascular permeability (Busse 1998). In addition, following exposure to inhaled antigen, airway macrophages secrete IL-1, TNF- α and IL-6 (via Fc ϵ RII) which acts on epithelial cells to induce their release of GM-CSF, IL-8 and RANTES, and thereby recruitment of inflammatory cells. Secretion of chemokines (e.g., eotaxin) and cytokines (e.g., IL-4, IL-5) by basophils and mast cells may also attract more T cells, APCs, and effector cells, such as eosinophils, to the site of insult.

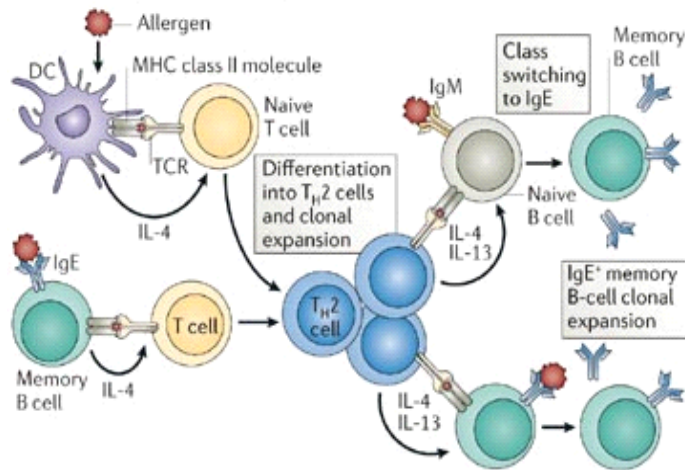
Simultaneously, DCs within the mucosa present allergen to T cells (Guermonprez, Valladeau *et al.* 2002), and within 6 to 48 hours (LAR), additional T cells are recruited to the area (Busse 1998). The expression of Fc ϵ RI on the surface of DCs greatly facilitates their uptake, processing, and presentation of allergens to T cells during the LAR. Furthermore, the cytokine milieu created as a result of mast cell and basophil activation most likely drives a polarized Th2 response. Further production of Th2 cytokines at the local site augments the recruitment and activation of additional T cells and eosinophils. During the late phase, mediators released from mast cells, basophils, eosinophils, and T cells act in concert to induce the vascular changes, bronchoconstriction (via smooth muscle activation), and mucus changes observed during the LAR.

The chronic inflammation: Repetitive allergen challenge leads to persistent inflammation in the lungs. The cytokines and chemokines released by mast cells, macrophages and the T cells further increase trafficking of activated T lymphocytes into the airways, and these continue to release Th2 cytokines i.e. IL-4, IL-5, IL-9 and IL-13, thus recruiting yet more

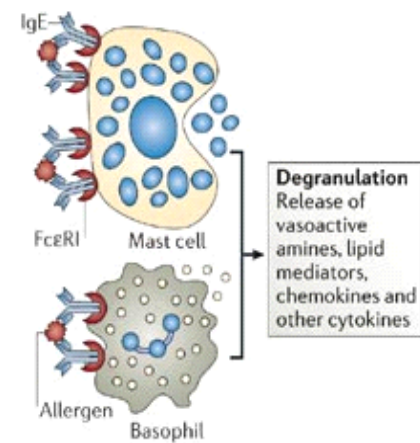
Figure 2.1 Pathogenesis of asthma (taken and modified; (Larche, Akdis *et al.* 2006).

(a) Sensitization to allergens and development of specific B-cell and T-cell memory. Differentiation and clonal expansion of allergen-specific Th2 cells leads to the production of cytokines (IL-4 and IL-13), which induce immunoglobulin class switching to IgE and clonal expansion of naive and IgE⁺ memory B-cell populations. T-cell activation in the presence of IL-4 increases differentiation into Th2 cells. (b) Early asthmatic reaction (EAR). Crosslinking of mast-cell and basophil cell-surface FcεRI (high-affinity receptor for IgE)-bound IgE by allergens leads to the release of vasoactive amines (such as histamine), lipid mediators, chemokines and other cytokines (such as IL-4, IL-5 and IL-13), and to the immediate symptoms of allergic disease. (c) Allergic inflammation (late phase of the allergic reaction, LPR). Following migration to sites of allergen exposure under the influence of chemokines and other cytokines, allergen-specific T cells are reactivated and clonally expand. This further recruits and activates eosinophils, mast cells and basophils, which release histamine, chemokines and other cytokines and perpetuate the inflammatory cascade.

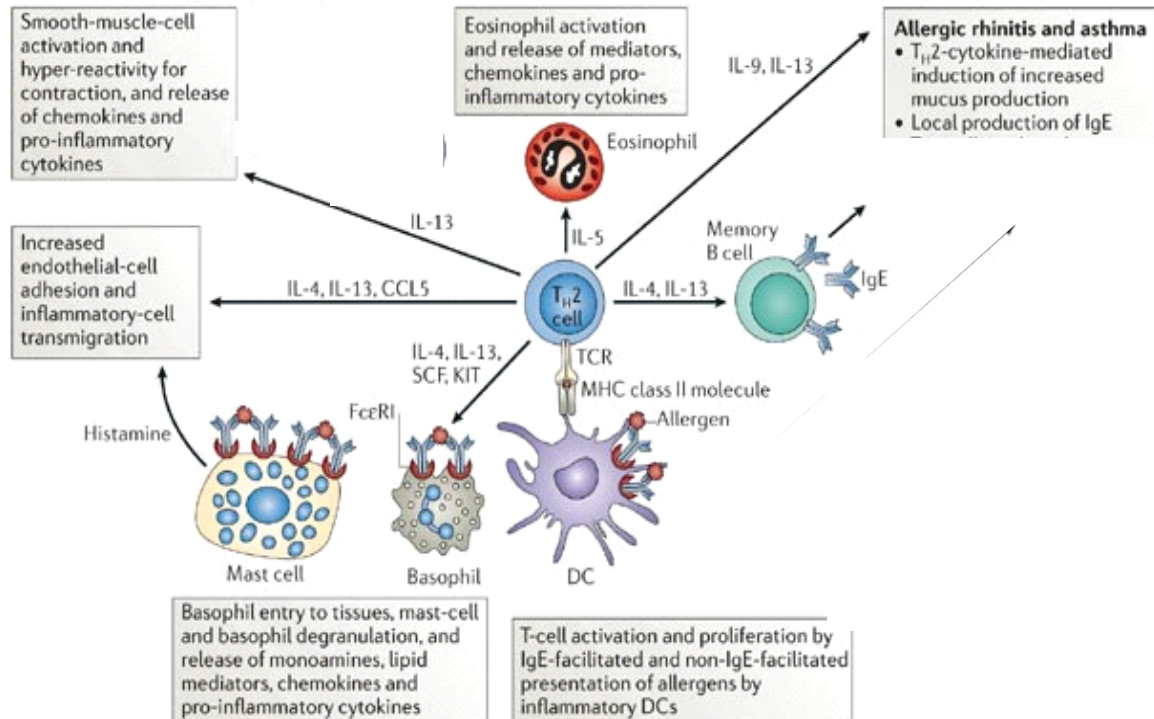
a Sensitization and memory induction



b Immediate phase: type 1 reaction



c Late phase: allergic inflammation



inflammatory cells sustaining the inflammatory cascade (Medoff, Thomas *et al.* 2008). Once the inflammatory reaction is initiated, eosinophil infiltration further contributes to tissue damage by the release of major basic protein, eosinophil cationic protein, leukotrienes and other inflammatory mediators (Wardlaw, Brightling *et al.* 2000). The repetitive cycle of tissue damage and inflammatory cell recruitment becomes chronic with time. Even in the absence of sustained allergen, the chronic inflammation persists. Other immune cells that are recruited to the lung wall during the late response as a result of T-cell activity are neutrophils and basophils. Neutrophils, eosinophils, and other cells involved in the inflammatory response produce alterations in the structure and function of resident mucosal cells such as smooth muscle, fibroblasts, and epithelial cells. Chronic airway changes include wall thickening, subepithelial fibrosis, increased mucus production and goblet cell mass, myofibroblast hyperplasia, and epithelial cell hypertrophy which cause bronchoconstriction and AHR. The state of chronic and relapsing airway constriction may further lead to permanent remodeling of the airways. The amplified inflammation and therefore the impaired airflow associated with it, is thought to persist as long as an increased number of leukocytes is maintained in an activated state in the airways. Thus, asthma is an immunoinflammatory disease in which T cells orchestrate the pathologic events. The details of specific roles of each cells in asthma pathogenesis is discussed below.

2.1.2 Cellular mechanisms in asthmatic inflammation

Over the past 100 years, different models have been proposed to explain the pathophysiology of this complex disorder. The most widely accepted is that airway inflammation drives the asthma phenotype (Walter and Holtzman 2005). A new paradigm of asthma causation is based on the observation that inflammation develops in individuals who manifest both

increased susceptibility to epithelial injury or impaired healing and Th2 allergic sensitization. The early and late asthmatic responses occur as a result of interactions between resident structural and immune cells which together orchestrate an inflammatory cell influx, culminating in an amplified and prolonged airway inflammatory cascade, airflow limitation and eventually airway remodeling (Ferreira 2004). An understanding of these complex interactions is important to the development of novel approaches to asthma therapeutics. The focus of this section of this thesis will be on various cells involved in asthmatic responses and their respective roles in asthma pathobiology.

2.1.2.1 Mast cells and Basophils

Mast cells are major players in responses to the various signals of innate and acquired immunity, and thus can undergo immediate and delayed release of inflammatory mediators. The unique anatomic position of the mast cells in the mucosa, submucosa and alveolar walls (Schwartz 1998) allows them to participate in lung allergic reactions. Mast cells constitute 0.25-0.5% of the total nucleated bronchoalveolar lavage (BAL) cells and 3-5% of the cells from enzymatically-dispersed lung in normal subjects (Forsythe and Ennis 1998). compared with the seven-fold elevated mast cell numbers seen in asthmatic patients (Koshino, Arai *et al.* 1996).

It is believed that mast cells are the critical trigger during episodes of acute asthma (Broide, Gleich *et al.* 1991). The inhaled allergen interacts with mast cells on the luminal surface of the airway epithelium, stimulating their release of histamine and other mediators, thus increasing epithelial permeability by disruption of epithelial tight junctions. This allows penetration of allergen to the submucosal mast cells and other cells (Norn and Clementsen 1988). Activation of these cells via cross linking of the high affinity IgE receptor (FcεRI) leads to the

release of both preformed (e.g. histamine, peroxidase, chemotactic factors and neutral proteases) and newly generated (e.g. cytokines, sulfidoleukotrienes (LTs) and prostaglandins (PGs)) mediators. These mediators are responsible for the symptoms of the immediate response. The preformed mast cell mediators induce acute bronchoconstriction, vasodilation, vascular leakage and increased mucus secretion while the *de novo*-synthesized mediators in addition establish a critical milieu in which the late phase inflammatory response can proceed (Busse 1998).

Preformed histamine stored in the granules is released within 5-30 minutes of FcεRI aggregation, although both spontaneous as well as IgE-mediated histamine release has been reported to be increased in BAL mast cells of asthmatic persons (Flint, Leung *et al.* 1985). Prostaglandins D₂ (PGD₂) and F_{2α} (PGF_{2α}) are potent bronchoconstrictors which, when inhaled at subthreshold contractile concentrations, increase airway AHR in asthmatic subjects (O'Byrne 2002). The cysteinyl leukotrienes (LTC₄, LTD₄, LTE₄), which can cause bronchoconstriction with up to 1000-fold greater potency than histamine, bind to receptors on the smooth muscle cells (Griffin, Weiss *et al.* 1983). Cysteinyl leukotrienes (CysLTs) cause smooth muscle cell proliferation and hypertrophy (Porreca, Di Febbo *et al.* 1996). Thus Bradding *et al.* (Bradding, Walls *et al.* 2006) suggested that the presence of mast cells within airway smooth muscle (ASM) may mediate ASM hypertrophy and hyperplasia, and the smooth muscle dysfunction that is related to AHR and variable airflow obstruction.

Mast cells may further participate in late asthmatic response and in chronic inflammatory changes via elaboration of a number of cytokines, including IL-4, IL-5, IL-6 and TNF-α (Bradding, Roberts *et al.* 1994). Mast cell generation of IL-4 may be an important factor in orienting uncommitted T cells towards a Th2 phenotype (Chomarat and Banchereau 1998) and may thereby promote Th2 cells secretion of IL-5 thus setting the stage for eosinophilic

inflammation (IL-5 is an eosinophil differentiation factor). Mast cells orchestrate infiltration of eosinophils not only through inducing up-regulation of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells but also through release of eosinophil-chemotactic factors like platelet activating factor (PAF) and LTB₄. In addition they enhance eosinophil survival by release of GM-CSF and IL-5 (Resnick and Weller 1993). In IgE-mediated reactions, mast cells provide an immediate preformed source of TNF- α , which has multiple pro-inflammatory effects, including leukocyte trafficking via enhanced adhesion molecules and chemokine expression as well as enhanced AHR (Thomas 2001). TNF- α also provides important maturational signal for DCs involved in development of immune responses (Cumberbatch, Fielding *et al.* 1994), and activates alveolar macrophages and neutrophils for inflammatory mediator production (Sirois, Menard *et al.* 2000). Thus, mast cells clearly provide a link between the early asthmatic phase and the late inflammatory phase.

2.1.2.2 Macrophages

The alveolar macrophages (AMs) are found in the alveolar spaces and conducting airways and can be retrieved by BAL. They are multifunctional cells, capable of either enhancing or suppressing inflammatory responses according to their microenvironment (Peters-Golden 2004). AMs express the low affinity IgE receptor (Fc ϵ RII), and do so at increased levels in asthmatic subjects (Williams, Johnson *et al.* 1992). Evidence as reviewed by Lane *et al* suggests that the total number of peroxidase stained -positive AM obtained from BAL fluid are increased at 48 and 96 hrs after allergen challenge (Lane, Sousa *et al.* 1994) .

Activation of AMs through Fc ϵ RII signaling causes release of the pro-inflammatory cytokines IL-1 and TNF- α as well as leukotrienes (Lane, Sousa *et al.* 1994). And AMs produce

substantial amounts of LTB₄, a potent chemoattractant for neutrophils and eosinophils and a neutrophil activator (Wenzel 2003). On comparison with healthy subjects, the AMs of atopic asthmatics produce more IL-1, IL-6, TNF- α and IL-10 and less IL-12, explaining their preferential induction of Th2 responses. Expression of granulocyte/macrophage colony stimulating factor (GM-CSF) is increased in the airways of asthmatics; approximately 65% of GM-CSF-positive airway cells are macrophages (Lane, Sousa *et al.* 1994). GM-CSF expression in the lung not only amplifies eosinophilic inflammation but also enhances MHC-II expression on both monocytes and AMs (Hawrylowicz, Caulfield *et al.* 2001), which could increase their antigen-presenting capacity. On the other hand, in some systems, AMs are poor antigen-presenting cells and actively suppress T cells responses (Holt 1993). But the presence of GM-CSF could potentially reverse the immunosuppressive effects of AMs (Bilyk and Holt 1993). Furthermore, the production of IL-10, a potent anti-inflammatory cytokine which inhibits both antigen-presenting and T cell functions, is reduced in monocyte and AMs of asthmatic individuals (Borish, Aarons *et al.* 1996). AMs also release monocyte chemotactic protein-1 (MCP-1), which causes recruitment and activation of additional monocytes and macrophages in the lung (Lane, Sousa *et al.* 1994), and MCP-4, a potent recruiting signal for eosinophils and lymphocytes. Thus, AM which are otherwise immunosuppressive under homeostatic conditions possibly lose their suppressive function under inflammatory conditions and further amplify asthmatic responses (Bilyk and Holt 1993; Takabayshi, Corr *et al.* 2006)

2.1.2.3 Neutrophils

The role for neutrophils in asthmatic inflammation has been recently recognized. There exists a strong association between neutrophilic inflammation and severe asthma, corticosteroid

dependent asthma, asthma exacerbations, nocturnal asthma, asthma in smokers, occupational asthma and sudden onset fatal asthma (Kamath, Pavord *et al.* 2005). Neutrophils release a variety of inflammatory mediators including lipids (LTB₄, PAF, thromboxane A₂ [TXA₂], LTA₄, cytokines [IL-1 β , IL-6, IL-8, TNF, TGF- β], proteases [elastase, matrix metalloproteinase (MMP-9), collagenase], microbiocidal products [i.e. lactoferrin, myeloperoxidase (MPO), lysozyme], reactive oxygen intermediates and nitric oxide [NO]), which together can cause airway epithelial injury, mucus hyper secretion and airway obstruction (Sampson 2000). Allergen challenge is an important trigger for neutrophil influx, and 4 hrs after segmental allergen (focused to specific part of bronchial tree) challenge the increases in neutrophil numbers correlated with IL-8 expression, levels of which are allergen dose-dependent (Nocker, Out *et al.* 1999). The increased numbers of neutrophils in lung autopsy specimens from patients who died suddenly from asthma suggests their participation in the acute asthmatic inflammatory response (Sur, Crotty *et al.* 1993). Significantly, increased numbers of induced sputum neutrophils have been found in severe asthmatics compared to mild asthmatics and normal controls even in the absence of recent infections. Interleukin (IL)-8 and MPO levels (both indicators of neutrophil activation) were also increased in asthmatics with highest levels found in severe asthma (Jatakanon, Uasuf *et al.* 1999). Woodruff *et al* suggested that chronic severe asthma may be attributed to either chronic airway infection with viruses or atypical bacteria or that chronically activated airway epithelial cells hypersecrete neutrophil chemoattractants as IL-8 (Woodruff and Fahy 2002). However, it is still unclear whether neutrophilia occurs as a primary pathological process or is secondary to high dose corticosteroid therapy (which prolongs neutrophil survival) and needs further investigation.

2.1.2.4 Epithelial Cells

The bronchial epithelium, being the primary interface for inhaled antigens, plays a key role in interactions between the external environment and the internal milieu. Our thinking of its role from merely being a physical barrier has been transformed by the recognition of its playing a central role in modulating chronic inflammation and in the maintenance of mucosal integrity (Busse 1998). Epithelial cells can be activated by endogenous (IL-1, TNF, IL-4, IL-13, TGF) or exogenous (LPS, virus, air pollutants, proteolytic enzymes) agents (Takizawa 2005). In asthmatics, they bear FcεRI and FcεRII receptors suggesting that they can be activated directly by IgE-bound allergens (Campbell, Vignola *et al.* 1994; Campbell, Vachier *et al.* 1998).

Activated bronchial epithelial cells are the cellular source of a wide array of pro-inflammatory cytokines and chemokines like IL-6, GM-CSF, IL-8 (CXCL8), eotaxin (CCL11), RANTES (Regulated on Activation Normal T cell Expressed and Secreted; CCL5) and Thymus and Activation Regulated Chemokine (TARC). The CC chemokines, RANTES and eotaxin cause primarily eosinophil influx and also recruitment of other inflammatory cells characteristic of chronic airway inflammation (Takizawa 2005). The epithelial damage and desquamation in the airways of asthmatics has been attributed to basic proteins released by activated eosinophils. The levels of major basic protein (MBP) and eosinophil-derived neurotoxin are elevated in BALF of asthmatics (Broide, Gleich *et al.* 1991). Bronchial and alveolar type 1 epithelial cells constitutively express intracellular adhesion molecule-1 (ICAM-1), the levels of which are increased in asthmatic patients (Bentley, Durham *et al.* 1993), and this is suggestive of heightened capacity for recruitment, localization and activation of neutrophils, eosinophils and lymphocytes. The increased expression in asthmatics of thymic stromal lymphopoietin (TSLP), produced at the epithelial interface, correlates with the expression of the chemokine

TARC/CCL17 which play a role in chemoattracting Th2 cells into the airways thus further amplifying the inflammatory cascade (Ying, O'Connor *et al.* 2005) .

2.1.2.5 T lymphocytes

Lymphocytes play pivotal roles in activation and establishment of chronic and persistent inflammatory processes in asthma (Busse 1998). Peripheral blood lymphocytes of patients experiencing acute exacerbations of asthma show marked elevations in T cell activation markers, including the IL-2 receptor (CD25), class II HLA-DR and a very late activation antigen (VLA-1) compared to control subjects (Arm and Lee 1992). In addition, increased numbers of activated T cells found in BAL fluid of asthmatics correlate positively with eosinophil recruitment into the airway wall, a characteristic of chronic airway inflammation (Bradley, Azzawi *et al.* 1991). A critical role for Th2 cells in asthma has been widely accepted. Polarized Th2 cells preferentially express the receptors CCR3, CCR4 and CCR8 and thereby interact with their ligands, i.e. eotaxin, monocyte-derived chemokine (MDC) and TARC (Larche, Robinson *et al.* 2003). Ying *et al* demonstrated increased eotaxin and CCR3 expression in bronchial biopsies of asthmatics (Ying, Robinson *et al.* 1997), and the increased expression of CCR4 and its MDC and TARC ligands on airway epithelium after allergen challenge suggests possible lymphocyte recruitment to asthmatic lungs (Panina-Bordignon, Papi *et al.* 2001).

The increased expression by T cells of IL-4, IL-5 and GM-CSF in the bronchi of asthmatics emphasizes the central role for Th2 lymphocytes in the pathogenesis of this complex disorder (Robinson, Hamid *et al.* 1992). As reviewed previously, IL-4 is a switch factor for IgE production, up-regulates vascular cell adhesion molecule -1 (VCAM-1; enabling thereby eosinophil recruitment), and is a Th2-differentiation factor (O'Byrne, Inman *et al.* 2004). Studies

from IL-4 knock-out (KO) mice revealed no Th2 cytokine secretion by T cells recovered from airways, and this correlated with the absence of inflammatory airway changes (Chung and Barnes 1999). Experiments with anti-IL-4 antibody experiments suggests that treatments given before sensitization prevent allergic inflammation, but those given after priming and before allergen challenge are not effective in preventing sequelae to allergic inflammation. Together, this indicates a role for IL-4 in the initiation and development of Th2-polarized allergen-specific immune responses. On the other hand IL-13 has been implicated in the effector phase of the immune response, such that it is sufficient to induce AHR and sub-epithelial fibrosis (Wills-Karp 2004). Interestingly, a recent study showed that, independent of IL-13, IL-4 can still induce AHR and goblet cell hyperplasia, suggesting that an IL-13–dominant induction of these effects reflects either a greater production, persistence, or potency of IL-13 than IL-4 (Perkins, Wills-Karp *et al.* 2006).

Other cytokines secreted by T cells include IL-3, IL-5 and GM-CSF (Busse 1998). IL-5 plays an important role in the development, differentiation, activation, recruitment and survival of eosinophils (O'Byrne, Inman *et al.* 2004), all of which are important to late asthmatic phases (see section 2.1.2.6 below). IL-9, a T cell growth factor, increases in expression after allergen provocation in asthmatics (Erpenbeck, Hohlfeld *et al.* 2003) and IL-9 has been shown to promote eosinophil maturation in synergy with IL-5 (Louahed, Zhou *et al.* 2001). In addition IL-9 transgenic mice display significantly enhanced eosinophilic airway inflammation, elevated serum total immunoglobulin E, and AHR following lung challenge (McLane, Haczku *et al.* 1998) thus suggesting its important role in asthma pathogenesis.

Thus, T cells act as important initiators and perpetuators of asthmatic inflammation through elaboration of various cytokines. Depletion of CD4⁺ T lymphocytes with anti-CD4

monoclonal antibody eliminates both eosinophilia and airway reactivity (Gavett, Chen *et al.* 1994). Thus, T cells are the critical mediators of asthmatic inflammation but so far, asthma therapies available have not been able to address this immunoinflammatory cause.

2.1.2.6 Eosinophils

Blood and lung eosinophilia is a cardinal feature of bronchial asthma (Arm and Lee 1992). Following allergen challenge, eosinophils appear in BAL fluid during the late response and are associated with decreases in peripheral eosinophil counts and appearance of eosinophil progenitors in the circulation (Busse 1998). Biopsy studies reviewed by Wardlaw *et al* confirmed that eosinophils were enriched by 100-fold (relative to neutrophils) in the airway submucosa of asthmatic subjects (Wardlaw, Brightling *et al.* 2000). Eosinophils develop from the bone marrow under the influence of IL-3, GM-CSF and late differentiation factor, IL-5, each of which are produced by T lymphocytes (Wardlaw, Brightling *et al.* 2000). Originally, it was believed that IL-5 alone stimulated eosinophil egress from the bone marrow. Overproduction of IL-5 in transgenic mice results in profound eosinophilia, while deletion of the IL-5 gene causes a marked reduction of eosinophils in the blood and lungs after antigen challenge (Rothenberg and Hogan 2006). However, eotaxin has also been shown to stimulate eosinophil mobilization from the bone marrow of guinea pig femurs (Palframan, Collins *et al.* 1998). Eosinophil migration depends on the cooperative actions of chemotactic and priming signals. It has been demonstrated that eotaxin-evoked eosinophil accumulation *in vivo* is upregulated by co-administration of IL-5 (Collins, Marleau *et al.* 1995). In addition double eotaxin-1/IL-5 gene deleted mice show greater suppression of eosinophilic recruitment to the lung and abolition of AHR associated with reduced IL-13 production (Mattes, Yang *et al.* 2002). Thus, IL-5 increases the pool of eotaxin-

responsive cells and primes them to respond via chemokine receptor-3 (CCR3), which is expressed at high levels on eosinophils. In addition to the eotaxins, other chemokines, such as RANTES, monocyte chemoattractant protein -3 (MCP-3), MCP-4 and macrophage inflammatory protein-1 α (MIP-1 α), can each bind to the CCR3 receptors and are chemotactic for eosinophils. The fact that IL-4 and IL-13 are potent inducers of eotaxins (via STAT-6) support that T cells are critical to allergic inflammation (Menzies-Gow and Robinson 2001; Rothenberg and Hogan 2006).

The extravasation of eosinophils from the circulation into the asthmatic airways is a tightly regulated process. The signals generated at the site of allergic inflammation activate both endothelial cells and circulating leukocytes to express adhesion molecules (Arm and Lee 1992). Endothelial cells express P (Platelet)-selectin, ICAM-1 (Intercellular Adhesion Molecule-1) and VCAM-1 (Vascular Cell Adhesion Molecule-1) on stimulation with cytokines such as IL-4, IL-13 and TNF. Eosinophils express P-selectin glycoprotein ligand-1 (PSGL) by which they bind to P-selectin (Wardlaw 1999). ICAM-1, which is the ligand for leukocyte function-associated antigen-1 (LFA-1) and Mac-1, binds to both neutrophils and eosinophils and VCAM-1 binds selectively to eosinophils via VLA-4 receptor (Bochner, Luscinskas *et al.* 1991). Wardlaw suggested a multistep model for eosinophil trafficking. The capture step (tethering) seems to be important in selective eosinophil migration through cooperative effects of PSGL-1/P-selectin and VLA-4/VCAM-1 in mild to moderate asthma, whereas in asthma exacerbations VLA-4/VCAM-1 play dominant receptor-mediating roles in eosinophil capture. In the activation and arrest stage, eosinophils up-regulate their surface receptors in response to chemoattractants, such that eosinophil adhesion and transmigration is mediated by VLA-4/VCAM-1 and β 2 (CD18) integrins binding to ICAM-1 (Wardlaw 1999).

BAL eosinophils recovered 19 hr after segmental bronchial allergen challenge was found to be hypodense compared to control subjects. Hypodense eosinophils are activated, expressing increased IgG and complement receptors, and producing inflammatory mediators (e.g. thromboxane B₂) in response to stimuli (Kroegel, Liu *et al.* 1994). The cytokines IL-3, IL-5 and GM-CSF can activate eosinophils. Both Fc γ R and Fc ϵ R are expressed on the eosinophil surface, such that their binding by IgG and IgE respectively, induces eosinophil degranulation (Seminario and Gleich 1994).

Mucosal damage in asthma seems to be caused by cytotoxic and proinflammatory mediators released by activated eosinophils. The basic proteins which are stored in distinctive secondary granules are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN), and all are toxic to bronchial epithelial cells (Wardlaw, Brightling *et al.* 2000). MBP is present in the sputum during severe asthma and has been localized by immunofluorescence to sites of respiratory epithelial damage (Filley, Holley *et al.* 1982). MBP is also capable of activating basophils, mast cells, neutrophils and platelets. ECP, a neurotoxin, has been shown to be toxic to guinea pig trachea, and is present in the submucosa of patients who died from asthma (Arm and Lee 1992). EPO functions both as a cationic toxin in the absence of hydrogen peroxide and as a peroxidase in the presence of hydrogen peroxide (Gleich, Adolphson *et al.* 1993). The combined EPO, hydrogen peroxide and halide enzyme system induces substantial damage to the airway epithelium and induces histamine release from airway mast cells (Davis 2002).

Eosinophils also produce PAF, which is a chemotactic factor (for eosinophils and neutrophils) and powerful bronchoconstrictor. Eosinophils also preferentially synthesize LTC₄, another potent mediator causing bronchoconstriction and changes in vascular permeability

(Gleich, Adolphson *et al.* 1993). Furthermore, eosinophils express mRNA for cytokines such as transforming growth factors (TGF- α , TGF- β , IL-1, GM-CSF, and IL-5, and they secrete IL-1, GM-CSF, and IL-3 thus affecting other cells as well their autoactivation (Gleich, Adolphson *et al.* 1993). The experimental models of airway inflammation in which mice are gene-targeted to remove eosinophils showed structural changes compared to wild type mice thus suggesting key role of eosinophils in airway remodeling (Humbles, Lloyd *et al.* 2004; Lee, Dimina *et al.* 2004; Kay 2005). Anti-IL-5 treatments cause a reduction in bronchial mucosal eosinophils and are associated with decreased levels of TGF- β in BAL fluid, suggesting that eosinophil-derived TGF- β may regulate lung tissue modeling. Thus, eosinophils are not only central effector cells in chronic asthmatic inflammation, but may also contribute to airway remodeling (Flood-Page, Menzies-Gow *et al.* 2003) and their activation and recruitment depends greatly on T cells.

2.1.3 AIRWAY HYPERRESPONSIVENESS (AHR)

AHR or airway hyperreactivity is the hallmark feature of asthma and is defined as an increased ability of the airways to constrict in response to non-specific stimuli, such as histamine, methacholine, leukotrienes, chemicals and pharmacological or physical stimulation. It is important to measure the severity of AHR, as it is strongly correlated with severity of asthma (i.e. inflammation) and therefore with the treatment levels required for the existing symptoms. Multiple independent and interdependent pathways mediate the development of AHR in allergic inflammation. Airway narrowing may be caused by changes in neural tone, airway epithelial damage, airway wall edema, mucus hypersecretion and smooth muscle hypertrophy, each of which may be attributed to various inflammatory cells and mediators. On the other hand, AHR

can be induced even in the absence of inflammation. Thus, it is very important to dissect the underlying mechanisms in order to intelligently design appropriate treatment.

2.1.3.1 AHR, nerves and neuropeptides

The lungs and airways are innervated by a complex system of autonomic nerves with afferent and efferent effector functions. Three types of nerves control airway function. The parasympathetic neurons in the vagus nerve play important roles in neural bronchoconstrictor mechanisms of the airway. The main neurotransmitter released by this system is acetylcholine (Ach) which acts via muscarinic receptors (M1, M2 and M3). The airway smooth muscles express M3 receptors that cause bronchoconstriction, while mucosecretion is mediated by both M1 and M3 receptors expressed by submucosal glands. The M2 receptors are inhibitory type receptors that are expressed on cholinergic nerves and negatively regulate the release of Ach. Evidence suggests that in animal models of AHR and asthma there is increased release of Ach (Larsen, Fame *et al.* 1994).

The sympathetic nervous system does not affect the airways directly but influences the cholinergic tone of the airway smooth muscles. Adrenaline and noradrenalin are the neurotransmitters that mediate their effect through stimulation of α or β receptors. The β_2 receptors are important in asthma as they mediate bronchodilatory effects by relaxation of smooth muscle (Rhoden, Meldrum *et al.* 1988). Another important system involved with neural airway control is the non-adrenergic non-cholinergic (NANC) system, which is described as excitatory (e-NANC) or inhibitory (i-NANC). The e-NANC consists of non-myelinated sensory C fibres and mediates their bronchoconstriction effects through the release of neuropeptides substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) (also called tachykinins), and

calcitonin gene related peptide (CGRP). These tachykinins bind to neurokinin receptors NK1 (found on blood vessels), NK2 (expressed on smooth muscle cells) and NK3 (expressed on cholinergic nerves), respectively. Increased levels of SP and NKA have been detected in the airways of asthmatic patients (Howarth, Springall *et al.* 1995; Heaney, Cross *et al.* 1998), and inhalation or intravenous administration of NKA leads to bronchoconstriction in asthmatic subjects (Groneberg, Quarcoo *et al.* 2004). The i-NANC system mediates bronchodilatory effect through release of vasoactive intestinal peptide (VIP) and nitric oxide (NO). There are various mechanisms (explained below) by which neural control of the airways can be altered.

2.1.3.2 AHR and inflammation

Airway inflammation is currently considered an important part of the complex mechanisms causing AHR (Walter and Holtzman 2005). It has been suggested that AHR is a key indicator for inflammation (Gibson, Saltos *et al.* 2000). On the contrary the nonatopic subjects who never experienced asthma but had quadriplegia displayed increased AHR in response to methacholine without evidence of airway inflammation (Grimm, Chandy *et al.* 2000). In addition, it has been shown that sensitization of mice with limiting doses of Ovalbumin (OVA) can induce AHR in the absence of airway inflammation (Schneider, Li *et al.* 2001). Various inflammatory cells and mediators have been implicated in AHR. Degranulating mast cells release histamine, prostaglandins (PGD₂), leukotrienes (LTC₄, LTD₄ and LTE₄), TXA₂, PAF, etc, which potently increase airway responsiveness in asthmatics compared to normal subjects. Mast cells also release proteases (e.g. tryptase) which signal to neurons via proteinase activating receptor-2 (PAR2) to stimulate the release of neuropeptides SP and CGRP (Steinhoff, Vergnolle *et al.* 2000). Evidence suggests a role for mast cells in AHR, as mast cell-deficient mice

pretreated with anti-IgE do not develop AHR (Martin, Takeishi *et al.* 1993), but, on the other hand, IgE-deficient mice can develop AHR and airway inflammation, suggesting that mechanisms other than IgE mediated mast cell degranulation may be induce AHR (Mehlhof, van de Rijn *et al.* 1997).

Eosinophils are the main effector cells of chronic asthmatic inflammation (Saeed, Badar *et al.* 2002; Rothenberg and Hogan 2006) and eosinophilia is of course related to AHR, but whether eosinophilic inflammation causes AHR is still a contentious issue. As reviewed above, eosinophils secrete a myriad of mediators (e.g., leukotrienes, PAF and eosinophil basic proteins) which are strong bronchoconstrictors. The latter mediators are cytotoxic to airway epithelial cells, and epithelial damage exposes sensory nerve endings to external stimuli. These then release neuropeptides which cannot be degraded (due to loss of enzyme production by damaged epithelium) and hence causes enhanced airway responses (Wills-Karp 1999). In addition, MBP has been shown to potentiate contraction of airway tissues *in vitro* without causing epithelial damage. Furthermore, the degree of M2 receptor dysfunction is related to the number of eosinophils surrounding the airway nerves (Fryer, Adamko *et al.* 1999). MBP has been shown to cause smooth muscle hyperreactivity by competitively inhibiting binding of Ach to M2 receptors, thus increasing release of Ach and hence AHR (Wills-Karp 1999). Recently Lee *et al* showed using eosinophil lineage knock-out (PHIL) mice that following OVA sensitization and allergen challenge there was no mucus accumulation or AHR, suggesting an essential role of eosinophils in AHR and asthma pathology (Lee, Dimina *et al.* 2004). IL-5 gene knock-out or anti-IL-5 neutralizing antibody suppresses eosinophilia and AHR, while IL-5 gene over-expression enhances these parameters (Sugita, Kuribayashi *et al.* 2003). On the contrary, eosinophilic inflammation can occur without AHR, as eosinophilic bronchitis patients who have

never experienced asthma symptoms, develop eosinophilic inflammation on allergen challenge, but not AHR (Brusasco, Crimi *et al.* 1998). Humbles *et al* created eosinophil-depleted mice by manipulation of the GATA-1 promoter and found that, following asthma sensitization, AHR and mucus secretion were unaffected (Humbles, Lloyd *et al.* 2004). Similarly, anti-IL-5 suppressed eosinophilia but not AHR in asthmatic atopic patients (Sugita, Kuribayashi *et al.* 2003). Allergen-driven Th2 responses also develop in the absence of eosinophils (Humbles, Lloyd *et al.* 2004). While a role for eosinophils in asthmatic pathology and to some extent in AHR is evident, other contributing factors may also be operative in AHR. The Th2 cytokine IL-13 is a central mediator in induction of AHR in the allergic lung (Wills-Karp, Luyimbazi *et al.* 1998). In addition, the role of undefined genetic components has been suggested in different strains of mice in the development of AHR irrespective of inflammatory input (Wilder, Collie *et al.* 1999). The variation in the association between eosinophil and development of AHR may be because of the differences in degranulation of eosinophils in different model systems or the probable role of IL-5 in release of tachykinins which is independent of its effect on eosinophils (Wills-Karp 1999).

2.1.3.3 AHR and smooth muscle

Smooth muscle connects the cartilaginous rings in the larger airways whereas it encircles the smaller airways, and thus the constriction of the latter causes more severe airway narrowing than the former (Ingram 1991). And equivalent degrees of airway smooth muscle (ASM) shortening, will have greater effect in airways with thickened (i.e., versus normal) walls (Hogg 1993) as occurs in the bronchi of the patients dying of asthma (Schmidt and Rabe 2000). Such increases in the ASM may be attributed to hypertrophy and/or hyperplasia. The hypertrophic

response might result from mechanical restraint or pressure or volume stimulus, whereas hyperplasia may result from inflammatory mediators such as histamine, tryptase, PDGF, endothelins, etc. that bathe the smooth muscles of the asthmatic airways (Black and Johnson 1996). Cytokines such as IL-1 and IL-6 in the asthmatic airways can also potentiate ASM proliferation (Wills-Karp 1999). Smooth muscle contraction is dependent on intracellular calcium levels and activation of myosin light chain kinase (MLCK), and cytokines such as IL-1 β and TNF- α modulate calcium signaling in ASM exposed to contractile agonists (Schmidt and Rabe 2000). In addition, IL-1 and TNF- α alter β -adrenoceptor signaling mechanisms, thus impairing the relaxation of smooth muscle (Wills-Karp 1999). IL-5 or GM-CSF can also cause increased responses to Ach in rabbit airways (Schmidt and Rabe 2000).

In conclusion, a complex phenomenon such as AHR, where a myriad of interacting mediators, cytokines and cells are involved, is difficult to be attributed to a single specific causative agent.

2.1.4 Immunoglobulin E and G: Role in asthma pathobiology

2.1.4.1 IgE antibody and its receptor

Immunoglobulin E (IgE), discovered 40 yrs ago by K. and T. Ishizaka (Kuby 2006) plays an important role in hypersensitivity reactions, and presently, including both early and late phase asthmatic reactions (Wills-Karp 2003). IgE has the shortest half-life of the immunoglobulins (2.5 days) and much of it is found in the tissues. In normal healthy individuals, the serum concentration of IgE is \approx 150 ng/ml. In parasitized or atopic individuals the concentration of IgE can spike as high as 1000 ng/ml (Gould, Sutton *et al.* 2003; Wills-Karp 2003). B cell switching to IgE synthesis is a two-signal process; signal-1 is provided by Th2 cytokines IL-4 and IL-13,

which activate transcription at the IgE-specific locus, while the second signal is a B cell-activating signal that involves engagement of CD40 on B cells by CD40 ligand (CD40L) on T cells, resulting in deletional switch recombination (Prussin and Metcalfe 2006). Mast cells and basophils can be important sources of IL-4 and IL-13 (Bradding, Roberts *et al.* 1994; Devouassoux, Foster *et al.* 1999) and perhaps CD40L (Schonbeck and Libby 2001), so could provide both signals, but this would likely play greater roles in IgE amplification rather than in IgE induction. Furthermore, IgE responses in IL-4-deficient mice were found to be restored by reconstitution with T cells and not non-T cells (Schmitz, Thiel *et al.* 1994) suggesting importance of T cells in generating antibody responses.

IgE binds to effector cells through the high affinity FcεRI receptor and/or the low affinity FcεRII receptor (i.e. CD23). The FcεRI receptor is abundant in mast cells and basophil membranes (200,000 molecules/cell) but it is expressed at lower levels on peripheral blood DCs, monocytes and Langerhan cells (Wills-Karp 2003). Ligation of FcεRI by cross-linking of IgE-allergen complexes on mast cells triggers release of preformed and newly generated mediators, and this induces mucosal edema, mucus production and smooth muscle constriction in bronchial mucosa. Allergen activation of mucosal mast cells may also foster interaction with local cells expressing CD40 (e.g., B cells) and, in the presence of IL-4 and IL-13, induce IgE class-switch and thereby enhance local IgE synthesis (Gould, Sutton *et al.* 2003). When targeted to FcεRI on DCs through binding with allergen-specific IgE, allergens can effectively reduce the threshold of the DCs allergen recognition, allowing lower doses of allergen to efficiently trigger T cell stimulation. In addition, higher levels of FcεRI are found on the lung DCs of atopic asthmatics relative to healthy subjects. Thus, IgE is a positive regulator of its receptors (Yamaguchi, Lantz *et al.* 1997).

2.1.4.2 IgG antibodies

Immunoglobulin G (IgG) antibodies can mediate hypersensitivity reactions in animal models, but this has not been validated in humans. In normal healthy individuals the serum concentration of IgG is 10 mg/ml. IgG (primarily IgG₁) mediates its action through activation receptors FcγRI, FcγRIIA and FcγRIIIA and inhibitory receptor FcγRIIB (Ravetch and Bolland 2001). Evidence suggests that anaphylactic reactions can be triggered through IgG (Fc-specific) mediated mast cell activation in IgE deficient mice (Oettgen, Martin *et al.* 1994). Mast cells express IgG₁ FcRs RIIB and RIII. Attenuation of passive anaphylaxis induced by IgG₁ in Fcγ chain-deficient and FcγRIII-deficient mice while enhanced anaphylaxis in FcγRIIB-deficient mice suggests importance of IgG₁ and its receptor in mast cell activation *in vivo* (Ravetch and Bolland 2001). On the other hand, in human subjects the risk factor for asthma is allergen sensitization or IgE antibodies and the presence of IgG (allergen-specific total IgG and IgG₄) antibodies are not associated with development of asthmatic response (Platts-Mills, Vaughan *et al.* 2001; Platts-Mills 2001).

In conclusion, both IgE and IgG₁ in the mouse model and IgE in human subjects are important triggers for allergic inflammation in the airways. In animal models IgE has been suggested to transfer both acute and late phase reaction to allergen challenge (Babu and Arshad 2001; Platts-Mills 2001). In clinical studies anti-IgE therapy thus has potential for attenuating both the early and late phase responses (Fahy, Fleming *et al.* 1997).

In brief, on allergen exposure, DCs take up the allergen and migrate to the draining lymph nodes where they present antigen to T cells, polarizing them towards Th2 type cells. The latter secrete IL-4 and IL-5, which are required for IgE production and for recruitment and maintenance of eosinophils respectively. On subsequent allergen exposures, IgE-activated mast

cells release an array of mediators that effect the immediate allergic reaction, whereas in the late phase reactions Th2 cells (by elaboration of cytokines and chemokines and further inflammatory cell recruitment) drive the immunopathology.

2.1.5 Asthma therapeutics

The goal of asthma therapeutics is minimization of symptoms, restoration of lung function as well as reversal of pathological changes within the airways. Some currently available asthma therapies are highly effective in controlling the asthmatic symptoms when used correctly. However, asthma is a disease involving abnormal immunological function, such that recent research strategies are being directed to addressing the immunoinflammatory basis of the disease.

2.1.5.1 Pharmacological agents in asthma treatment

Conventional treatments that have been used are primarily symptomatic in nature, targeting either the AHR (e.g., bronchodilators) or inflammatory responses (e.g., steroids) (Lin and Casale 2002). More refined approaches have included anti-IgE (Finn, Gross *et al.* 2003), -cytokine, cytokine receptor Ab (Tomkinson, Duez *et al.* 2001; Foster, Hogan *et al.* 2002; Leckie 2003), leukotriene antagonists (Ducharme, Hicks *et al.* 2002; Asakura, Ishii *et al.* 2004) or Th1 skewing unmethylated cytosine-guanine dinucleotides (i.e. CpG motifs) (Jain, Kitagaki *et al.* 2003).

Bronchodilators constitute an important part of asthma management, such that β_2 -adrenoreceptor agonists are the most effective drugs for symptom relief. They induce smooth muscle relaxation by increasing intracellular levels of cyclic adenosine monophosphate and

opening potassium channels (Rabe and Schmidt 2001). In addition, some studies show that they may have mast cell-stabilizing effects and thereby inhibit airway microvascular leak (Barnes 1993). But evidence suggests that regular use of salbutamol can augment airway CXCL8/interleukin-8 responses to allergen challenge which could contribute to the airway inflammatory response (Gordon, Swystun *et al.* 2003). Some studies suggest that drug tolerance and worsening of the underlying inflammation (masked by the relieving effects of bronchodilation) cause delay in seeking treatment and thereby worsening of the unmanaged condition. As per the guidelines, short-acting inhaled β_2 -agonists should only be used on demand and for prevention of exercise-induced bronchospasm, and long-acting inhaled β_2 -agonists (LABAs) should only be used as required as adjunctive therapy with inhaled corticosteroids in patients whose asthma is not controlled with low to medium doses of the inhaled corticosteroid (Kelly 2005). Methylxanthines such as theophylline are non-selective phosphodiesterase inhibitors which, being effective both in early and late asthmatic responses, are used as an adjunct therapy with corticosteroids. Their use is limited due to their non-selective inhibition of PDEs (Rabe and Schmidt 2001).

Corticosteroids (CS) are the mainstay of modern asthma management because of their anti-inflammatory effects. Long term use of systemic corticosteroids is limited by side effects, such as adrenal suppression and withdrawal symptoms (Rabe and Schmidt 2001), but inhaled corticosteroids are the drugs of first choice in long-term asthma treatment. Corticosteroids bind to the glucocorticoid receptor (GR) localized in the cytoplasm of target cells, which then translocate to the nucleus where it binds DNA-specific sequences (glucocorticoid response elements) resulting in altered transcription (Barnes 1993). Corticosteroids increase eosinophil apoptosis, decrease mast cell numbers and inhibit mediator expression by macrophages and

cytokine release by T cells (Barnes 1993). Corticosteroids, not only inhibit T cell migration but appear to arrest DCs in an immature state, characterized by retained endocytic activity and reduced expression of MHC II, costimulatory receptors, and T cell-activating cytokines such as IL-12 (Georas 2004). It has also been suggested that CSs also inhibit GM-CSF dependent uptake of antigen by DCs, but not the antigen (pre-processed) presentation *per se* (Holt and Thomas 1997). Due to concerns with safety issues, CSs with high local anti-inflammatory properties such as ciclesonide which were earlier under trial (Taylor, Jensen *et al.* 1999) have now been shown to inhibit T cell migration and cytokine production (Heijink, Kauffman *et al.* 2007; Magnussen, Hofman *et al.* 2007).

Mast cell stabilizers such as cromolyn and nedocromil sodium have anti-inflammatory effects attributable to their inhibition of mediator release by mast cells, but not the bronchodilator effect and are used for prophylactic treatment (Barnes 1993). Anti-leukotrienes are the class of drugs that inhibit either LT synthesis by inhibition of 5-lipoxygenase or via their interaction with LT-receptors. They significantly improve lung function by causing bronchodilation as well as preventing eosinophil accumulation and are used as add-on therapy with inhaled corticosteroids (Rabe and Schmidt 2001; Ducharme, Schwartz *et al.* 2004).

2.1.5.2 Allergen-Specific Immunotherapy (SIT)

SIT is disease modifying, rather than palliative. It involves subcutaneous or more recently sublingual injections of gradually increasing quantities of specific allergens over time to an allergic patient, until a dose is reached that induces active immunity to the allergen, thereby minimizing symptomatic expression of the disease as well as reducing medication usage (Larche, Akdis *et al.* 2006). Successful SIT is associated with increases in allergen-specific IgG4

antibodies, which block the allergen's epitopes access to FcεRI-bound IgE and thereby prevents allergen engagement by IgE thus reducing IgE-induced mast cell degranulation (Larche, Akdis *et al.* 2006), but also IgE-facilitated allergen presentation mediated by CD23 (van Neerven, Wikborg *et al.* 1999). In contrast, if allergen-specific IgG and IgE are directed against distinct allergen epitopes the IgG will not block the IgE-dependent response (Denepoux, Eibensteiner *et al.* 2000). It has been suggested that in the absence of pro-inflammatory signals, as occurs in SIT, allergen presenting DCs are modulated into IL-10- producing cells, which induces T-regulatory cell differentiation, thus inhibiting inflammatory responses (Larche, Akdis *et al.* 2006). SIT also modulates T cell responses through induction of both T cell anergy and IL-10-producing T regulatory -1 (Tr1) cells and/or CD4⁺ CD25⁺ regulatory T cells in sufficient numbers to down-regulate the allergic response (Till, Francis *et al.* 2004; Schmidt-Weber and Blaser 2005). It is the only treatment available for specific allergy but is not very effective in patients with multiple sensitivities, which is the usual case (Adkinson, Eggleston *et al.* 1997). Long-term duration of therapy along with uncertainty to achieve tolerance adds to its disadvantages.

2.1.5.3 Immunomodulatory Treatment

As reviewed earlier, IgE is an important mediator of asthmatic inflammation in the airways and can transfer early and late phase reactions, making it an important target for asthma therapeutics. Moreover, anti-IgE has been shown to attenuate early and late phase responses in clinical trials (Fahy, Fleming *et al.* 1997). Recently, US Food and Drug Association (FDA) has approved humanized monoclonal anti-IgE antibodies such as Omalizumab for use in patients refractory to the treatment with corticosteroids. It is directed against the receptor-binding domain of IgE, thus preventing IgE binding to FcεRI receptors on mast cells and basophils and inhibiting

IgE and allergen-dependent degranulation and mediator release. As FcεRI receptors are expressed on DCs and CD23 on B cells, it has been suggested that anti-IgE might interfere with antigen presentation to T cells (Avila 2006). So far the clinical trials done indicate that Omalizumab reduces inflammation, provides symptomatic relief and reduces steroid use in patients with allergic asthma (Buhl 2005). However, Omalizumab does not improve airway hyperreactivity, but is used as add-on treatment with corticosteroids (Busse, Corren *et al.* 2001; Djukanovic, Wilson *et al.* 2004). High cost, injection site reactions, risk of anaphylaxis are significant side-effects associated with anti-IgE therapy (Avila 2006).

The critical role of cytokines in allergic inflammation has led to the development of strategies to modulate cytokine activity as a novel therapeutic approach for asthma. Since IL-4 has the ability to drive Th2 differentiation, it is an attractive target for inhibition. Numerous IL-4-blocking agents, such as soluble IL-4 receptors, anti-IL-4 antibodies, IL-4 mutein (binds to and blocks IL-4Rα and IL-13Rα1), anti-IL-4 receptor antibody and STAT-6 inhibitors interfering with downstream IL-4 signaling have been tried. Due to conflicting findings, some of the treatments have been withdrawn while others need further investigation (Barnes 2003). Although IL-4 antagonism has been shown to be effective in humans and mice (Henderson, Chi *et al.* 2000; Steinke and Borish 2001), it has been shown to be unnecessary for the maintenance of memory T cells (Yamashita, Shinnakasu *et al.* 2004) which might explain its' less than optimal efficacy.

IL-5 mediates eosinophilic inflammation, such that anti-IL-5 therapy can reduce AHR and eosinophilia in asthma-phenotype guinea pigs (Mausner, Pitman *et al.* 1993). In human trials with a monoclonal anti-IL-5 (Mausner, Pitman *et al.* 1993) the subjects showed reductions in sputum and circulating eosinophils but not reduced AHR or late phase responses (Leckie, ten

Brinke *et al.* 2000). Similarly, blood and sputum, but not tissue eosinophilia, can be reduced without inhibiting AHR (Flood-Page, Menzies-Gow *et al.* 2003). Anti-IL-13 effectively suppresses lung inflammation and partially suppresses airway remodeling, goblet cell hyperplasia, and subepithelial fibrosis. Anti-IL-13 had little effect on AHR (Kumar, Herbert *et al.* 2004) in one study, but in another study it was found to be effective (Yang, Volk *et al.* 2004). A soluble form of the IL-13 receptor, IL-13R α 2, has been used as a decoy receptor for secreted IL-13 and was found to be effective in blocking IgE generation, pulmonary eosinophilia and AHR in mice (Wills-Karp, Luyimbazi *et al.* 1998). As reviewed by O'Byrne, soluble TNF- α receptor-IgG₁Fc fusion protein (i.e. Etanercept) therapy improves lung function and methacholine AHR in severe asthmatics and in patients unresponsive to steroids (O'Byrne 2006). Nevertheless, overall, current evidence suggests that targeting individual cytokines in asthma will not be an appropriate therapeutic approach.

Immune deviation (one of the aspects of which is skewing Th2 to Th1) has recently been proposed as a viable therapeutic option. Gavett, O'Hearn *et al.* showed that administration of IL-12, a regulator of Th1 cell responses, to rats inhibited allergic inflammation. In human trials IL-12 treatments resulted in a significant decrease in blood and sputum eosinophil counts, but had no such effect on allergen-induced AHR or late asthmatic responses (O'Byrne 2006). Interferon- γ (IFN- γ) inhibits Th2 cells, but administration of nebulized IFN- γ to asthmatic subjects did not reduce eosinophilic inflammation (Boguniewicz, Martin *et al.* 1995) despite the fact that it ameliorated inflammation in animal models (Barnes 2003). It has also been suggested that increasing biological responsiveness to Th1-promoting cytokines like IL-12 and IL-18 (resulting in enhanced IFN- γ production) by using chemically modified allergen glutaraldehyde-polymerized OVA prevents Th2-responses without necessarily changing expression of the

cytokines themselves (Lewkowich, Rempel *et al.* 2005). Unmethylated cytosine-guanine dinucleotides (i.e. CpG motifs) are enriched in bacterial DNA. They specifically interact with Toll like receptor-9 (TLR-9) and, through upregulation of costimulatory molecules, stimulate the production on IL-12 and IL-18 by APCs and IFN- γ by NK cells, and thereby induce a strong Th1-biased response (Wohlleben and Erb 2006). Numerous reports confirm the protective effect of CpG-ODNs given with, or conjugated to allergen in acute and chronic allergic inflammation and airway remodeling (Jain, Kitagaki *et al.* 2003). Evidence suggests that CpG-ODNs inhibit the Th2 responses by induction of IFN- γ and IL-10 (Kitagaki, Jain *et al.* 2002). These effects are associated with, but not dependent on Th1 responses, as studies have confirmed the anti-allergic effects of CpGs are independent of IFN- γ and IL-12 (Kline, Krieg *et al.* 1999). Since Th2 responses to inhaled allergen recur as little as 4 weeks after a single dose of CpG DNA (Broide and Sriramarao 2001), imposing limitation to its use. Furthermore, Th1 responses stimulate delayed type hypersensitivity or cellular immunity which does not occur in healthy subjects and is likely not desirable as a therapeutic outcome.

2.2 DENDRITIC CELL IMMUNOBIOLOGY

DCs are a family of antigen-presenting cells (APC) whose ability to process and present antigens makes them crucial in regulating immune responses. The decision whether or not encounter with an antigen will lead to an immune response is in some respects controlled at the level of APC and is subject to tight regulation (Lambrecht, Pauwels *et al.* 1996). DCs located in the peripheral tissues, such as mucosal surfaces, skin, spleen etc, form an efficient detection system for foreign antigens (Steinman 1991).

2.2.1 Dendritic cells (DC) in the lung

DCs develop from hemopoietic precursors. They are broadly classified as either conventional DCs, which includes both the myeloid and lymphoid subsets, or plasmacytoid DCs (pDCs). Human DC1 are analogous to the conventional DCs in mice, while in the human DC2 subset is analogous to pDCs (Grayson 2006). Two models have been suggested with regards to generation of functionally distinct subsets of DC. The functional plasticity model suggests that all DC come from a single hematopoietic lineage, and that specific subsets represent different activation states, which arise under local environmental conditions. On the other hand, the specialized lineage model emphasizes separate developmental lineages that produce specialized and functionally committed DC subsets. But Shortman *et al* have suggested that a mixture of these two models may be operative, and have proposed functional plasticity as a general feature (Shortman and Liu 2002).

DCs are distributed throughout the lung and can be found in the airway wall, lung parenchyma, alveolar surface, visceral pleura and pulmonary vascular bed. In most species, the steady state density of DCs in the airway epithelium ranges from 500-1000 per mm². The half-life of lung DCs ranges from 1.5-2 days in the airways to 3-4 days in the periphery, and these turnover rates increase markedly in inflammatory conditions (Stumbles, Upham *et al.* 2003; Grayson 2006). The DCs in the conducting airways of mice are of myeloid origin, expressing CD11c, MHC II and CD11b, and low to intermediate levels of CD205 (DEC-205). The origin of the DC in the conducting airways of mice is unknown, but they are irregularly-shaped cells comparable to blood-derived DCs located in the epithelial and subepithelial tissue of bronchi and bronchus-associated lymphoid tissue (BALT) (de Heer, Hammad *et al.* 2005). The interstitial DCs in mice and humans are immature CD11c⁺ cells of myeloid origin, express low levels of

costimulatory markers like CD40, CD80, CD86, and have a longer half-life of about 10 days. The same is true for human alveolar DCs (de Heer, Hammad *et al.* 2005). On the other hand, mouse alveolar DCs, which are CD11c⁺, CD11b⁺ and F4/80⁺, have been shown to retain antigen for about 8 weeks in situ, which could in part explain the chronic inflammatory nature of asthma (Julia, Hessel *et al.* 2002). Recently, a plasmacytoid DC (pDC) population was identified in the human enzymatic lung digests characterized by their unique phenotype (i.e., IL-3 receptor (CD123)⁺, blood-derived cell antigen-2 (BDCA-2)⁺, CD11c⁻ with no expression of maturation markers (Demedts, Brusselle *et al.* 2005). Similarly, in mice, pDCs found in enzymatic lung digests are CD11c^{dim}, positive for the granulocyte marker Gr-1, B cell marker B220 and 120G8, and secrete IFN- α on exposure to virus (de Heer, Hammad *et al.* 2005)

In mouse lymphoid tissues, at least five subsets of DC have been described based on expression of MHC class II, CD11b, CD205, CD4, and CD8 (Shortman and Liu 2002). However, in the mouse respiratory tract (RT), a large population of CD11c^{high}CD4⁻CD8⁻CD11b⁺CD205⁺ "myeloid" DC (the most likely DC population involved in trafficking from the RT to the draining lymph nodes), a small pDC-like population of CD11c^{int}I-A^{d low}120G8^{pos} cells (Asselin-Paturel, Brizard *et al.* 2003)) and a prominent population of CD11c^{neg}120G8^{pos} cells was observed in both lung and airways (von Garnier, Filgueira *et al.* 2005). In addition to these DCs, a CD11c^{high}I-A^{d low}CD2^{pos} macrophage population is found in the lung parenchyma, and these have a potentially suppressive activity, and therefore could play an important role in immunological homeostasis (von Garnier, Filgueira *et al.* 2005).

Culture of mouse bone marrow in GM-CSF/IL-4 results in expansion of myeloid DC (Lutz, Kukutsch *et al.* 1999), and similar culture of human CD14⁺ blood monocytes give rise to CD11c⁺ HLA-DR^{low} E-cadherin⁻ langerin⁻ (i.e., myeloid) DC (Sallusto and Lanzavecchia 1994).

In the absence of inflammatory signals these DCs remain immature in phenotype, while on addition of maturational signals such as LPS or TNF- α , they become morphologically and functionally mature (Lutz, Kukutsch *et al.* 1999).

2.2.2 DCs, antigen presentation and peripheral surveillance

DCs continuously patrol the respiratory mucosal surfaces for inhaled foreign as well as endogenous antigens. This process includes (i) a constant immigration of the DCs to the lung, (ii) their capacity to take up and process these antigens and (iii) their transport and efficient presentation to T cells in the draining lymph nodes, and ideally culminates in the induction of appropriate immune responses (Vermaelen and Pauwels 2005). Under steady-state conditions, DC or their precursors are constantly recruited from the blood to the lung (Holt, Haining *et al.* 1994). DC homeostasis in lymphoid organs is maintained by migration of DC precursors (through the blood) which mature into DCs, divide a few times and then die, leaving a void to be replenished by bone marrow precursors (Liu, Waskow *et al.* 2007). It has also been suggested that DC are continuously replaced through successive cell divisions of antigen-bearing precursor cells that pass antigen molecules onto their progeny thus maintaining antigen presence in the population (Diao, Winter *et al.* 2007). Under inflammatory conditions, increased number of lung DCs are recruited to lung through the chemokine RANTES, macrophage inflammatory protein-1 α (MIP-1 α) and MIP-3 α which are secreted by epithelium and chemoattract immature DCs expressing CCR1, CCR5 and CCR6 receptors (Vermaelen and Pauwels 2005). The capacity of the DC to capture and process antigens is highly dependent on their stage of differentiation. Immature DCs have an immense capacity to phagocytose particles and microbes (Henderson, Watkins *et al.* 1997). DCs extend their pseudopodia into the airway lumen and capture antigen

there in, processing these into small immunogenic peptides. Immature DCs can take up antigen very efficiently by macropinocytosis or endocytosis via mannose, CR3, Fc γ R, Fc ϵ R, Toll-like receptors, DC-SIGN i.e. DC-specific intercellular adhesion molecule [ICAM]-3-grabbing nonintegrin receptors. Deslee *et al* recently illustrated the role of the mannose receptor in house dust mite (i.e., *Der p 1*) allergen uptake by monocyte derived-DCs and showed that this process is more efficient in dust mite-sensitive patients compared to healthy donors (Deslee, Charbonnier *et al.* 2002). As noted above in secondary responses, IgE and Fc ϵ R1-mediated allergen uptake is another important mechanism of allergen presentation which may lower the antigen recognition threshold of atopic patients and possibly perpetuate allergic tissue inflammation (Lambrecht, Pauwels *et al.* 1996; Hammad 2003). Once DC takes up the antigen the process of antigen processing and cellular maturation starts.

Chemokine receptors involved in homing of DCs to peripheral tissues (primarily CCR1, CCR5, and CCR6) are down-regulated (Luster 2002) allowing the maturing DC to escape from the tissue and enter the lymph flow. Associated with this change is up-regulation of CCR7, which allows the dendritic cell to respond to CCL19/CCL21. CCL19 (MIP-3 β) and CCL21 are expressed by high endothelial venules of the draining lymph node. Thus, the DC attains the ability to hone in to the lymph node (Saeki, Moore *et al.* 1999). While expression of CCR5, which binds MIP-1 α , MIP-1 β and RANTES, is reduced as maturation progresses, the expression CCR7, a receptor for the lymphoid chemokines, stromal cell-derived factor-1 α (SDF-1 α ; a CXCR4 ligand), MIP-3 β (a.k.a EBI1 ligand chemokine, ELC) and CC chemokine 6Ckine (secondary lymphoid tissue chemokine, SLC) is enhanced (Austyn 2000; Grayson 2006). This indicates that mature DCs respond preferentially to chemokines expressed in T cell-rich areas of secondary lymphoid tissues (Lin, Suri *et al.* 1998).

Several other phenotypic changes occur as DC enter lymph nodes, such as increases in expression of costimulatory and antigen presentation-associated molecules, including CD80/CD86 (B7.1/B7.2), CD1, intercellular adhesion molecule-1 (CD54), lymphocyte function associated antigen 3 (LFA-3), and MHC class I and II expression (Reis e Sousa 2006). The DCs also undergo significant shape change, with development of prominent dendrites. While these phenotypic changes are occurring, the antigen taken up by the above-noted mechanisms accumulate in the acidic endocytic compartment, where they are proteolytically degraded into peptides of 13 or more amino acids. These are then loaded on newly synthesized or recycling MHC-II molecules, which are then transported to the DC plasma membrane for presentation. The phagocytic capacity of DCs decreases as the antigen presentation ability increases with maturation (Guermonprez, Valladeau *et al.* 2002).

DC may interact with any passing T cell in the lymph node, but the antigen-specific receptor on the T cell (the TCR) scans the allergen peptide located MHC molecules on the DC. On presentation of the appropriate antigen in the correct MHC context, the TCR will fully engage forming a well-characterized immunologic synapse between the T cell and dendritic cell, thereby activating the T cell (Grayson 2006). A naïve T cell becomes an effector cell by the signals it receives from the DCs. Signal 1 is delivered through the TCR when it engages an appropriate peptide-MHC complex, signal 2 is the co-stimulation signal through CD28 when it engages CD80 and/or CD86, and signal 3 is the signal from APC to T cells to differentiate later to an effector cell (Reis e Sousa 2006).

2.2.3 Dendritic cells and Th2 polarization

The environment in which the DC is stimulated, the type of stimulus present and importantly the origin of the DCs are known to play important roles in shaping the effects that DCs have on the differentiation of the naive T cells to Th1, Th2 or regulatory T cell phenotypes (Reid, Penna *et al.* 2000). Th1 cells secreting IFN- γ develop predominantly in response to intracellular pathogens (bacteria and virus) whereas Th2 cells producing IL-4, -5 and -13 develop in response to extracellular pathogens (parasites and allergens) (Grayson 2006).

Under resting conditions, lung DCs do not secrete IL-12, but do express IL-6 and IL-10 so that airway mucosal responses are Th2-biased (Constant, Brogdon *et al.* 2002). On the contrary, mouse skin DCs produce IL-12 in response to contact allergens and hence promote Th1 response (Grabbe and Schwarz 1998). It has been shown that injection into syngeneic mice of DC subsets pulsed *ex vivo* with a protein antigen, keyhole limpet hemocyanin, directs the development of distinct Th cells. Antigen-pulsed GM-CSF-treated CD8 α^- DCs induce a Th2-type response, whereas injection of similarly treated CD8 α^+ DCs leads to Th1 differentiation *in vivo* (Maldonado-Lopez, De Smedt *et al.* 1999). However, antigen-pulsed CD8 α^+ DCs (not treated with maturation or tolerogenic stimuli) have been shown to tolerize asthma in a mouse model (Gordon, Li *et al.* 2005). It has also been suggested that Th2 might be a default pathway, that is type 2 T cells would develop spontaneously in the absence of an IL-12 signal (STAT-4) that represses the Th2-polarizing transcription factor GATA-3 (Moser and Murphy 2000). In addition, it has been shown that splenic or bone marrow-derived DCs (both OVA-pulsed and GM-CSF-treated) when given locally (Lambrecht, De Veerman *et al.* 2000; Sung, Rose *et al.* 2001) or systemically (Graffi, Dekan *et al.* 2002) can sensitize Th2 cells for induction of airway eosinophilia and AHR, suggesting that the lung environment preferentially induces Th2-responses. Not only the specific tissue or organ environment, but also the antigen dosage, affinity

and length of response may also affect Th1/Th2 polarization (Rogers and Croft 1999). It has been shown that while high levels of lipopolysaccharide (LPS) results in Th1-responses, low-level LPS exposure provides a positive stimulus (signal through TLR-4) for induction of Th2-dependent immunity via affects on DCs (Eisenbarth, Piggott *et al.* 2002). Signals from the tissue microenvironment (e.g., epithelial cell-derived TSLP, PGE2, histamine, or β 2-agonists) can also polarize myeloid DCs to induce Th2 responses (Upham and Stumbles 2003).

On encounter with T cells, mature DCs present not only pathogen- derived antigens as MHC-peptide complexes (signal 1) and costimulatory molecules (signal 2) but also molecules that polarize the T cells (signal 3). For example, bacterial CpG-DNA motifs induce type 1 DCs (which expressed elevated levels of IL-12) promote Th1 cells, whereas helminth eggs or worms induce type 2 DCs (which had low IL-12 production) induce Th2 cells through expression of OX40L. Thus pathogens evoke host-protective immune responses by conditional priming of immature DCs (Kalinski, Hilkens *et al.* 1999; Reis e Sousa 2006).

2.3 IMMUNOLOGICAL TOLERANCE

Tolerance is defined as the acquisition of non-reactivity of immune system towards a particular antigen. The importance of tolerance towards self-antigens was first appreciated by Ehrlich and Morgenroth when they discovered that goats injected with red blood cells from another goat always produced haemolytic antibodies, whereas goats immunized with their own red blood cells produced no antibody response. They coined the term “horror autotoxicus” to describe this (reviewed by (Paul 2003)). Tolerance is fundamental to the normal functioning of the immune system, but we have known for sometime that it is also possible to induce tolerance to non-self antigens (e.g. through oral tolerance) (Lowney 1965). This changing conceptual

framework of tolerance came with organ transplantation studies (Billingham and Brent 1956; Scully, Qin *et al.* 1994) that provided compelling evidence that tolerance is an acquired state, and that mechanisms such as anergy (lymphocyte survival in an inactive form), immune privilege, activation induced cell death and dominant regulation (suppression) may be important. Thus immunological tolerance may be defined as a mechanism by which potentially pathogenic adaptive immune responses are prevented, suppressed or shifted to a non-pathological mode (Weiner 2001) without changing the Th1/Th2 balance. In the context of allergic disease, a poignant question in my own work is why a portion of the population is allergic but the rest are unresponsive to allergens i.e. they are tolerant. Three different mechanisms are of importance in allergen tolerance.

2.3.1 Immune Deviation

Although immune deviation in broader sense means deviating allergic immune response to any other protective response but for the purpose of this thesis, we have used it relative to Th1/ Th2 balance. With the recognition of Th1 and Th2 cells, it was thought that it was Th1 versus Th2-specific cytokines (i.e., IFN- γ versus IL-4, IL-5 and IL-13, respectively) that determined healthy versus allergic status. Some early reports in which limiting dilution analysis was performed to quantify the frequency of grass pollen-reactive IL-4- versus IFN- γ -producing cells in the peripheral blood of healthy and allergic subjects suggested that Th1-skewed responses predominated in healthy subjects (Li, Simons *et al.* 1996). This incited a search for methods to skew atopic/Th2 responses to a “protective” Th1 mode. Various strategies, including use of IL-12, IFN- γ and CpG-ODN motifs as skewing adjuvants were used, with limited success, as discussed above. Although CpG-containing immunostimulatory DNA sequences (ISS) have been reported to shut down Th2 responses quite effectively (Hessel, Chu *et al.* 2005) they have

been reported to reduce allergic lung inflammation for 4-6 weeks (Sur, Wild *et al.* 1999; Broide and Sriramarao 2001). Besides this, they do not inhibit IgE-mediated mast cell/basophil degranulation (Ikeda, Miller *et al.* 2003).

2.3.2 Anergy

As stated by Schwartz, T cell anergy is a tolerance mechanism in which the lymphocyte is functionally inactivated following an antigen encounter, although it remains alive for an extended period in a hyporesponsive state. Models of T cell anergy affecting both CD4⁺ and CD8⁺ cells fall into two broad (but not perfect) categories (Schwartz 2003). One is a state of growth arrest called T cell *clonal anergy*, and the other represents a more generalized inhibition of proliferative and effector functions, which is called *adaptive tolerance*, but is sometimes referred to by others as *in vivo anergy* (Perez, VanParijs *et al.* 1997).

The induction of an anergic state in Th2 cells represents an active process, and is mediated by modulation of APC i.e. by down regulating the T cell activating capacity of APC (Taams and Wauben 2000). The anergized cells can be either CD4⁺ or CD8⁺ (Sato, Yamashita *et al.* 2002) and they produce very little in the way of immunoregulatory cytokines as IL-2, IL-4, IL-10, IFN- γ or TGF- β . It has been reported that suppression by anergic T cells is mediated via the APC, either through modulation of the T cell-activating capacity of the APC (APC/T cell interaction), or by inhibition of T cells recognizing peptide/MHC in close proximity on the same APC (T/T cell interaction) (Taams, van Rensen *et al.* 1998). Evidence suggests that allospecific mouse T cell clones rendered unresponsive inhibit proliferation by other responsive T cells specific for the same alloantigens. This inhibition requires the presence of APC, such that the coculture of anergic T cells with bone marrow-derived DCs leads to profound inhibition of the ability of the DC to stimulate T cells subsequently with the same or a different specificity. The

affected DC expressed low levels of MHC class II, CD80 and CD86. (Vendetti, Chai *et al.* 2000). This suppression is not attributable to passive competition for ligands on the APC surface, IL-2 consumption, or cytolysis, and is not mediated by soluble factors or reversed by addition of neutralizing anti-IL-4, anti-IL-10, and anti-TGF- β antibodies. Once the APCs are affected, they themselves can directly tolerize naïve T cells (Taams, Boot *et al.* 2000). Anergic T cells have been shown to profoundly inhibit the proliferation of responsive T cells in an antigen-specific manner and to greatly reduce their secretion of IL-2, IFN- γ , IL-4, IL-10 and TGF- β (Chai, Bartok *et al.* 1999). Reversal of the anergy can be accomplished with human clones by the addition of IL-2 (Essery, Feldmann *et al.* 1988).

2.3.3 Regulatory /Suppressor T cells

2.3.3.1 Naturally occurring CD4⁺CD25⁺ T regulatory cells (Treg):

CD4⁺CD25⁺ Treg cells were first described in mice with potent regulatory functions (Sakaguchi, Sakaguchi *et al.* 1995; Sakaguchi, Sakaguchi *et al.* 2001) and similar population was also described in humans (Jonuleit, Schmitt *et al.* 2001).

CD25⁺ cells comprise 5% of thymic CD4⁺CD8⁻ T cells and rely on radio-resistant elements of the thymus such as epithelial cells for their selection (Bach and Francois Bach 2003). Evidence has been provided that CD25⁺ T cells emigrate from the thymus to populate the periphery after they are educated on medullary DCs during the process of specific selection (Shevach 2002). Phenotype markers have also been used to define and deplete Treg cells *in vivo*. When freshly isolated they proliferate poorly and express CD25 (IL-2R α) (Itoh, Takahashi *et al.* 1999) but CD25 is expressed on all recently activated cells (Annacker, Pimenta-Araujo *et al.* 2001). A number of other markers have been associated with human and/or murine Tregs, including cytotoxic T lymphocyte activation antigen (CTLA- 4; CD152), neuropilin-1 (Nrp-1),

glucocorticoid-induced tumor necrosis factor family-related receptor (GITR), the chemokine receptors CCR4 and CCR8, and CD62L (L-selectin), lymphocyte activation gene-3 (LAG-3) and integrin $\alpha E\beta 7$ (CD103) (Beissert, Schwarz *et al.* 2006). *In vitro* studies suggest that the regulatory effect requires cell-cell contact mechanism mediated at least in part by granzyme B (Gondek, Lu *et al.* 2005). Also, signaling through the inhibitory “costimulatory” molecule CTLA-4 has been shown to play an important role (Read, Malmstrom *et al.* 2000; Loser, Scherer *et al.* 2005). Once activated, Tregs directly suppress self-reactive effector cell functions in an antigen non-specific manner (Thornton and Shevach 2000) although influenza peptide-specific Treg cells have been reported (Jordan, Riley *et al.* 2000). $CD4^+CD25^+$ T cells depend on exogenous IL-2 but inhibit the proliferative responses of $CD4^+CD25^-$ T cells by suppressing the capacity of the responders to transcribe IL-2 (Thornton, Donovan *et al.* 2004).

Studies in animals suggest that signal transducer and activator of transcription 1 (STAT1) signaling and transcription factor FoxP3 might be involved in the development and function of Treg cells, and that a disturbed balance of $CD4^+CD25^+$ Treg cells occurs in the STAT1-deficient and Scurfy (mutation of FoxP3) mice (Brunkow, Jeffery *et al.* 2001; Nishibori, Tanabe *et al.* 2004). However, in humans expression of FoxP3 is reportedly a normal consequence of $CD4^+$ T cell activation, so cannot be used as an exclusive marker of $CD4^+CD25^+$ Tregs cells (Allan, Crome *et al.* 2007). Tregs expressing CTLA-4 (constitutively or induced) can condition DCs to produce IFN- γ , thereby inducing activation of indoleamine 2,3-dioxygenase (IDO), which causes tryptophan degradation resulting in apoptosis of the effector T cells (Fallarino, Grohmann *et al.* 2003). Recently, high expression of LAG-3 (a CD4-related molecule that binds MHC class II) was found on $CD4^+CD25^+$ T cells upon activation and $CD4^+CD25^+$ T cells isolated from LAG-3 $^{-/-}$ mice displayed reduced suppressor capacity (Huang, Workman *et al.* 2004).

The role of CD4⁺CD25⁺ Treg cells may be complex in allergic disease. A recent study reported no differences in numbers of circulating CD4⁺CD25⁺ T cells in normal versus asthmatic subjects, although their numbers were raised during acute asthma exacerbation (Shi, Li *et al.* 2004). In addition, no differences in the suppressive activity of CD4⁺CD25⁺ Treg cells were observed between normal and atopic asthmatics (Shi and Qin 2005). However, the CD25⁺ T cells from non-allergic, but not from allergic, donors suppressed proliferation and IL-5 secretion by autologous allergen-stimulated CD4⁺CD25⁺ T cells during symptomatic periods, when pollen counts were highest (Ling, Smith *et al.* 2004). This suggests that there were increases in numbers of CD4⁺CD25⁺ T cells numbers during asthma exacerbations, and possibly a functional impairment that disturbs the balance between tolerance and immunity. A recent report suggests reversal of AHR by CD4⁺CD25⁺Foxp3⁺Lag-3⁺CTLA-4⁺CD45RC⁺ T cells during chronic exposure of “asthmatic” rats to allergen aerosols, and that this may be mediated by CD86 down regulation on the airway DCs (Strickland, Stumbles *et al.* 2006). OVA-specific CD4⁺CD25⁺ T cells have been shown to suppress airway inflammation, but not AHR in response to inhaled OVA in mice (Hadeiba and Locksley 2003). Thus, evidence suggests Treg cells play important roles in dampening the allergic responses, and thus that methods to upregulate the function of these cells could provide promising results in asthma therapy.

2.3.3.2 Tr-1 regulatory cells

Tr1 cells are a subset of human and mouse IL-10-producing Treg cells. They produce high levels of IL-10 and TGF- β , moderate amounts of IFN- γ and IL-5 IL-2 or IL-4 (Levings, Bacchetta *et al.* 2002). T cells activated by splenic APCs in the presence of IL-10 develop a unique cytokine secretion profile (IL-10⁺IL-5^{+/-}IFN- γ ⁺TGF- β ⁺) and are capable of inhibiting the development of colitis induced in SCID mice by pathogenic CD4⁺CD45RB^{high} splenic T cells

(Groux, O'Garra *et al.* 1997). Tr1 cells can also be induced by repetitive stimulation of human CD4⁺ T cells *in vitro* in the presence of IL-10 (Levings and Roncarolo 2000). Tr1 regulate the function of naïve and memory T cells *in vitro* and *in vivo* and can suppress both Th1 and Th2 responses to tumors, alloantigens and pathogens (Roncarolo, Bacchetta *et al.* 2001). Tr1 cells express the 'gene' repressor of GATA-3' (ROG); GATA-3 is the promoter-enhancer element essential to Th2 responses (Yamashita, Ukai-Tadenuma *et al.* 2004). Regulatory T cells that are distinct from Tr1 and CD4⁺CD25⁺ Tregs can reportedly also be generated after culturing conventional CD4⁺CD25⁻ murine T cells in the presence of IL-10 and TGF- β (Chen, O'Shaughnessy *et al.* 2003). Besides this, evidence suggests that CD4⁺CD45RB^{low} Treg cells maintain tolerance, in part indirectly, through the differentiation of IL-10-secreting Tr1 cells in a mouse model of inflammatory bowel disease (Foussat, Cottrez *et al.* 2003). Repetitive stimulation with Ag-loaded APCs (Steinman, Turley *et al.* 2000) or chronic antigen stimulation (Chakraborty, Li *et al.* 1999) leads to emergence of CD4⁺ T cells that suppress naïve T cell responses via IL-10-dependent mechanism.

Mouse Tr1 clones derived by antigen stimulation in the presence of IL-10 could prevent Th2 sensitization and IgE production if adoptively transferred prior to sensitization (Cottrez, Hurst *et al.* 2000). Akdis *et al.* showed an increased frequency of allergen-specific IL-10-producing CD4⁺ T cells in the blood of nonatopic individuals versus atopics and, that these IL-10-producing T cells could specifically inhibit allergen-activated IL-4-producing T cells (Akdis, Verhagen *et al.* 2004).

2.3.3.3 Th3 regulatory cells

Weiner originally described Th3 cells as CD4⁺ T cells, which produce TGF- β and not IL-10, IL-4 or IFN γ following induction by low doses of oral antigen. They are closely associated

with gastrointestinal tolerance, as they provide help in IgA secretion (Weiner 2001). Our lab has already reported that in mice given low doses of allergen-alum conjugates the lung-infiltrating lymphocytes secrete IgA and TGF- β with no evidence of Th1/Th2 responses (Schneider, Li *et al.* 2001) which is similar to Th3-regulated profile. We have also found that very low (1 ng) doses of allergen-alum induce anergic tolerance in these animals (unpublished data from our lab). This contrasts with the situation with oral tolerance where low doses induce Th3 cells and high doses anergy or deletion (Weiner 2001) which suggests that Treg induction might depend on antigen dose or the specific compartment. It has been demonstrated that Th3 cells could suppress the activation of Th1-cell as well as Th2-cell clones (Weiner 1997).

2.3.4 Infectious Tolerance

With the development of new tolerogenic strategies in transplantation studies, in particular the use of non-depleting anti-CD4 monoclonal antibodies (Mab) (Waldmann and Cobbold 1998) much emphasis came to the role of T cell mediated suppression. It was originally described in mice made tolerant to transplantation antigens after administration of anti-CD4 antibodies. They developed a powerful form of immune regulation that not only suppressed antigen-specific naïve or primed CD4 or CD8 T cells, but also responses to closely linked antigens (linked suppression). This form of tolerance is mediated by CD4 cells which suppress subsequent generations of cells by a phenomenon termed infectious tolerance (Cobbold and Waldmann 1998; Waldmann, Adams *et al.* 2006). Evidence suggests that TGF- β /IL-2-stimulated CD4⁺CD25⁺ Treg cells can induce CD25⁻ T cells to differentiate into CD4⁺CD25⁺ cytokine-independent suppressor cells, which in turn induce other CD25⁻ cells to become CD4⁺CD25⁺ Treg cells in a TGF- β - and IL-10-dependent manner, thus maintaining the powerful circuit of immunoregulation by sustaining CD4 Treg cells (Zheng, Wang *et al.* 2004).

Two distinct subsets of CD25⁺ Treg cells have been reported, including integrin $\alpha 4\beta 7$ -positive cells which can convert CD4⁺ T cell into IL-10-producing Tr1-like cells, and integrin $\alpha 4\beta 1$ -positive Tr cells which induce TGF- β -producing Th3-Like cells (Stassen, Fondel *et al.* 2004). These integrins are homing receptors for cellular migration of T lymphocytes to mucosal surfaces and inflamed compartments, respectively. Therefore, it can be said that $\alpha 4\beta 1$ CD25⁺ Treg cells migrate *in vivo* to inflamed tissues where they inhibit T cell responses and $\alpha 4\beta 7$ CD25⁺ Treg cells migrate to mucosal tissues to prevent chronic mucosal inflammation. Another important aspect of infectious tolerance is that regulatory T cells can render regional DCs tolerogenic by inhibiting the costimulatory molecule (MHC-II, CD80, CD86, CD40 and IL-12) expression, such that the DC can no longer support efficient T cell stimulation (Vendetti, Chai *et al.* 2000; Min, Zhou *et al.* 2003). As mentioned above, CD4⁺CD25⁺ Tregs expressing CTLA-4 can also activate IDO expression in DCs, which lends to tryptophan catabolism and thereby activation of apoptosis in effector T cells (Fallarino, Grohmann *et al.* 2003).

2.3.5 Interleukin-10 (IL-10) and Tolerance

Interleukin-10 was originally described as a mouse Th2 cell factor that inhibited cytokine synthesis by Th1 cells (Fiorentino, Bond *et al.* 1989). However, neutralization of IL-10 in peripheral blood mononuclear cell (PBMC) cultures reconstitutes both type 1 and type 2 cytokine production and proliferative responses. Indeed, increasing evidence indicates that IL-10 also acts as an inhibitor of Th2 cell responses. In particular, IL-10 down regulates IL-5 production by human resting T cells and Th2 clones (Schandene, Alonso-Vega *et al.* 1994; Zuany-Amorim, Haile *et al.* 1995; Zuany-Amorim, Creminon *et al.* 1996). As reviewed by Hawrylowicz *et al.*, IL-10 modulates many cells and effector functions associated with allergic diseases, including inhibiting the activation of, and cytokine generation by, mast cells, inhibiting

survival and cytokine production by eosinophils, inhibiting activation of Th2 cells and maturation of DCs, and enhancing IgG to IgE ratio by immunoglobulin switching (Hawrylowicz and O'Garra 2005). Evidence suggests that non-atopic subjects have an increased frequency of IL-10-secreting allergen-specific T cells, relative to atopic individuals (Akdis, Verhagen *et al.* 2004).

In allergen specific immunotherapy (SIT), increased IL-10 production by Treg cells causes specific anergy in peripheral T cells and skews specific antibody production toward IgG4-isotype response. Bee-keepers who have become tolerant to repeated stings and stung non-atopic individuals, both display augmented T cell IL-10 response (relative to bee-allergic individuals), and this further support the contention that IL-10 regulates tolerance to allergen (Akdis, Blesken *et al.* 1998). In these individuals, IL-10 acts directly on the CD28 signaling pathway and this is an important mechanism leading to anergy. IL-10 inhibits the antigen-induced secretion of both Th1 and Th2 cytokines, including IL-2, IFN- γ , IL-4, IL-5 and IL-13, such that neutralization of IL-10 significantly increases T cell proliferation and both Th1 and Th2 cytokine production (Joss, Akdis *et al.* 2000). It has also been shown that T cells engineered to produce IL-10 can downregulate Th2-induced airway hyper reactivity and inflammation, and this strongly suggest that IL-10 plays an important inhibitory role in allergic asthma (Oh, Seroogy *et al.* 2002). In addition, adoptive transfer of pulmonary DCs from wild type IL-10(+/+), but not IL-10 knock out (-/-), mice previously tolerized by exposure to soluble respiratory antigen induces antigen-specific unresponsiveness in recipient mice (Akbari, DeKruyff *et al.* 2001). Recent evidence suggests that passive transfer of CD4⁺CD25⁺ T cells from normal, but not IL-10^{-/-}, mice into sensitized mice results in reduced AHR and inflammation and increases in IL-10 and TGF- β (Joetham, Takada *et al.* 2007). Similarly, pulmonary delivery of an IL-10 transgene to mice

before sensitization or during allergen aerosol exposure strongly suppresses Th2 reactivity and AHR via overall suppression of CD11c⁺ APCs of the lung (Nakagome, Dohi *et al.* 2005). It has also been shown that the direct T-cell suppression by IL-10 is mediated by SHP-1 (Dominant-negative src homology 2 domain-containing protein tyrosine phosphatase 1) induced inhibition of CD28 or ICOS costimulatory pathways (Taylor, Akdis *et al.* 2007). However, a disadvantage of using IL-10 by itself as a therapeutic is its relatively short biological half-life (Hawrylowicz and O'Garra 2005).

2.3.6 Dendritic cells, Interleukin -10 and Tolerance

Under quiescent conditions DCs are immature sentinel cells that are avidly endocytic (Sallusto, Cella *et al.* 1995). Under steady-state conditions DC critically define immunologic self and thereby prevent the induction of autoimmunity and chronic inflammation triggered by innocuous environmental proteins, and this is indicative of their tolerogenic state (Steinman and Nussenzweig 2002). Resting state CD8 α ⁺ DC have also been shown to have tolerogenic potential (Kronin, Fitzmaurice *et al.* 2001) and indeed antigen pulsed CD8 α ⁺ DC can partially reverse AHR and pulmonary immunoinflammatory responses after local passive transfer into asthma phenotype mice (Gordon, Li *et al.* 2005). Specific subsets of DCs may differentially affect the development of antigen-specific Treg cells. For example, in the respiratory tract, mature myeloid CD8 α ⁻ DCs that transiently produce IL-10 and express inducible co-stimulator (ICOS)-ligand can induce Th2 regulatory cells (secreting IL-4 and IL-10) that express GATA-3 and Foxp3 and mediate respiratory tolerance (Akbari, DeKruyff *et al.* 2001). In contrast, mature CD8 α ⁺ that have been activated with heat-killed *Listeria monocytogenes* produce both IL-10 and IL-12 and induce Th1 regulatory cells that expressed IL-10, IFN- γ , T-bet and Foxp3 and thereby protect against airway hyperreactivity (Stock, Akbari *et al.* 2004; Umetsu and DeKruyff 2006).

Following ligation of CD80/86, CD8 α^+ and B220 $^+$ populations of DC express indoleamine 2,3-dioxygenase (IDO), an enzyme that mediates T cell suppression through tryptophan catabolism (Mellor, Baban *et al.* 2003). IDO expression can also be induced in CD8 α^- DCs by exposing them to CTLA4-immunoglobulin, which mimics signaling by CTLA4-expressing Treg subsets (Fallarino, Grohmann *et al.* 2003; Mellor and Munn 2004). Although the presence of CD8 α^+ DCs has not been documented in the lung (von Garnier, Filgueira *et al.* 2005), the plasmacytoid DCs (pDCs) that are present are crucial for maintaining tolerance to inhaled harmless antigens. Indeed passive transfer of pDC blocks subsequent attempts at allergen sensitization (de Heer, Hammad *et al.* 2004). Splenic pDCs secrete high levels of IL-10 after activation and, when pulsed with antigen, they induce specific Treg cell development and tolerance (Wakkach, Fournier *et al.* 2003). It was shown that when influenza virus peptide-pulsed immature DCs are injected, IFN-secreting virus-specific IFN- γ virus CD8 $^+$ T cells virtually disappear from the blood stream and in their place appear IL-10-secreting T regulatory cells (Dhodapkar, Steinman *et al.* 2001). IL-10-producing DC isolated from respiratory lymph nodes can similarly induce antigen-specific anergic/regulatory cells (Akbari, Freeman *et al.* 2002), and it is well acknowledged that T cells activation is strictly dependent upon their interactions with DC (Lanzavecchia and Sallusto 2001). Indeed it has been postulated that the immunosuppressive effects of IL-10 on T cells are largely realized through the effects of this cytokine on DC (Beissert, Hosoi *et al.* 1995; De Smedt, Van Mechelen *et al.* 1997).

DCs express certain molecules (eg. IL-10, TGF- β) with potential to inhibit T cell proliferation, so that genetic engineering of DCs to express immunosuppressive cytokines or other molecules is, in theory, an attractive approach to enhance tolerance in the context of allograft rejection and possibly autoimmune disorders (Lu, Lee *et al.* 1999). Earlier trials

suggested that viral IL-10 (encoded by Epstein barr virus and homologous to mammalian IL-10) may have therapeutic potential in inhibiting undesired immune response to allo- or auto-antigens (Takayama, Nishioka *et al.* 1998). It was shown in a mouse model of colitis that IL-10 gene therapy can induce complete remission of disease (Lindsay, Ciesielski *et al.* 2001). IL-10 gene-modified DCs induce antigen-specific tolerance in an experimental autoimmune myocarditis model, probably related to NF- κ B pathway inhibition and downregulation of costimulatory molecules (Li, Liu *et al.* 2006). DCs, either at immature or semi-mature stages, can influence the differentiation of both naturally-occurring CD4⁺CD25⁺ Treg cells and adaptive, IL-10-secreting CD4⁺ type 1 (Tr1) cells either *in vitro* or *in vivo* (Vigouroux, Yvon *et al.* 2004; Battaglia, Gregori *et al.* 2006). It was reported some time ago that immature DCs differentiated with IL-10 for the last 2 days of culture show a strongly diminished capacity to stimulate CD4⁺ T cell responses in an allogenic mixed lymphocyte reaction. This clearly suggested that IL-10 can render immature DCs into tolerogenic DCs, which could be a useful therapeutic tool (Steinbrink, Wolfl *et al.* 1997) in treating autoimmune or allergic disease. A recent report has shown treatment with OVA-presenting, IL-10-differentiated DC inhibits subsequent sensitization to OVA and proliferation of OVA-specific T cells upon transferred into ^{OVA}DC_{IL-10}-recipient mice. Rechallenge with the antigen *in vitro* and *in vivo* could not alter the inhibitory effects of IL-10-treated DCs (Muller, Muller *et al.* 2002).

As the outcome of innocuous environmental antigen challenge is allergen tolerance in healthy (i.e. non-allergic) individuals, a tolerogenic role for DCs seems ideal outcome to asthma therapeutics. In addition, tolerance is a more desirable therapeutic approach than immune deviation because non-allergic reactions are characterized by the absence of inflammatory cells, including Th1 cells or a modified Th2 response (Platts-Mills, Blumenthal *et al.* 2000).

Respiratory DCs acquire antigen regularly and move to the draining lymph nodes for antigen presentation, but they secrete IL-10 as they do so (Stumbles, Thomas *et al.* 1998). And IL-10 secreting DCs are tolerogenic in as much as they induce the differentiation of IL-10-secreting Treg cells *in vivo* (Akbari, DeKruyff *et al.* 2001). Inflammatory signals in the airways can turn off this default IL-10 DC response and can induce the DCs to shift from a tolerogenic to an immunogenic mode (Holt and Stumbles 2000). Nevertheless, we hypothesize that tolerization is superior to simple Th2-Th1 immune deviation in asthma therapeutics.

3.0 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

Allergen-presenting interleukin-10 treated bone marrow-derived DCs can be used for therapeutic tolerization of the severe allergic lung disease in a mouse model of asthma.

3.2 Objectives

The main objectives of this study are:

1. To generate and characterize normal, tolerogenic or immunogenic DCs.
2. Test the capacity of IL-10-differentiated DCs (DC_{IL-10}) to induce functional tolerance in mouse model of allergic lung disease/asthma.
3. To test the effectiveness of one or more DC_{IL-10} treatments over time.
4. Characterize the complex mechanisms involved in DC-induced tolerance.

4.0 MATERIALS AND METHODS

4.1 Mice

Female BALB/c mice (6-10 weeks of age) were purchased from the University of Saskatchewan (U of S) Animal Resource Centre and housed in the animal facility of Western College of Veterinary Medicine, U of S. They were housed in plastic cages, fed a standard laboratory diet, and given food and water *ad libitum*. The animal care protocols were approved by the U of S Animal Care Committee in strict accordance with the guidelines issued by the Canadian Council on Animal Care. All mice were euthanized under halothane (MTC Pharmaceutical., Cambridge, ON) inhalant anesthesia by cervical dislocation.

4.2 Generation of bone marrow-derived dendritic cells (BM-DC)

The principle method for generating BM-DC with GM-CSF was adapted from previous publications (Inaba, Inaba *et al.* 1992; Scheicher, Mehlig *et al.* 1992; Lutz, Kukutsch *et al.* 1999) with some modifications. Bacteriological Petri dishes with a 100 mm diameter were used (Falcon, No. 1029/Becton Dickinson, Heidelberg, Germany). The cell culture medium (R10) employed was RPMI-1640 (Gibson, Grand Island, NY) supplemented with penicillin (100 U/ml; Sigma Chemical Co, Mississauga, ON), streptomycin (100 go/ml; Sigma), L-glutamine (2 mM; Sigma), 2-mercaptoethanol (50 μ M; Sigma), and 10% heat-inactivated, fetal bovine serum (FBS) (Gibco, Grand Island, NY). At day 0, $4-5 \times 10^6$ bone marrow leukocytes were seeded at 37°C into 100 mm dishes in 5 ml R10 medium containing 200 U/ml (= 20 ng/ml) recombinant mouse

granulocyte/ monocyte colony-stimulating factor (rmGM-CSF) (Biosource International Inc., California, USA). After day 3 another 5 ml of fresh R10 medium containing 200 U/ml rmGM-CSF was added to the plates. On days 6 and 8, half of the culture medium was collected from each plate, centrifuged, and the cell pellet resuspended in 10 ml fresh R10 containing 200 U/ml and 75 U/ml (7.5 ng/ml) rmGM-CSF and placed back in the original plates. The GM-CSF dose was reduced to minimize granulocyte growth. The day 10 culture non-adherent cells were collected by gentle pipetting, centrifuged at $300\times g$ for 10 min at room temperature, resuspended in 5 ml fresh R10, and transferred into fresh 20 mm plastic tissue culture dishes (Falcon, No. 1029/Becton Dickinson, Heidelberg, Germany) containing 75 U/ml rmGM-CSF alone (to generate immature DC), or along with either IL-10 at 50 ng/ml (for tolerogenic DC) or tumor necrosis factor- α (TNF- α) at 10 ng/ml (for mature DC). The cells were then cultured for 3 more days. On day 13, the cells were incubated for 1-2 hours at 37°C with grade V Ovalbumin (OVA; 50 μ g/ml; Sigma), and then the cells were washed two times. The individual DC populations were harvested separately and characterized as noted below.

4.2.1 Evaluation of Cell yields

Cultured cells were washed twice and a 10 μ l aliquot was mixed 1:1 with trypan blue solution (0.05% in saline; Sigma). Trypan blue-negative leukocytes (erythrocytes were excluded by size and shape) were counted as viable under the microscope (40x objective) using a Neubauer hemocytometer.

4.2.2 Fluorescence-activated cell sorter (FACS) analysis

Cultured cells ($2-4 \times 10^5$) were suspended in 100 μ l of R10 media and then 1 μ l of fluorescein isothiocyanate (FITC)-conjugated marker-specific or isotype-matched control antibody was added for 30 min on ice (4°C). These cells were then washed and resuspended in 100 μ l of fresh R10 media, then fixed by addition of 100 μ l of 1% paraformaldehyde, and stored at 4°C until analyzed. The samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA) within 48 hrs of the staining procedure. The mouse marker-specific FITC-labeled antibodies used were directed against mouse CD3 (pan-T cell marker; isotype rat IgG2b), CD4 (rat IgG2b), CD8 (rat IgG2a), CD11b (macrophage marker rat IgG2b), CD11c (DC marker hamster IgG1), CD14 (monocyte marker rat IgG1), CD16 (neutrophil marker rat IgG2b), CD19 (B cell marker rat IgG2a), CD25 (activated T cell marker rat IgM), CD40 (hamster IgM), CD45RB (rat IgG2a), CD54 (hamster IgG1), CD80 (hamster IgG2), CD86 (rat IgG2a), MHC-I (mouse IgG2a), MHC-II (mouse IgG2b) (PharMingen Canada Inc., Mississauga, ON), DEC-205 (DC marker) and F4/80 (macrophage marker rat IgG2b) (Serotec Ltd, Oxford, UK). Isotype controls were purchased from PharMingen Canada Inc.

4.2.3 Phagocytosis

The phagocytic capacity of the BM-DC was analyzed as described elsewhere (Sallusto, Cella *et al.* 1995; Lutz, Rovere *et al.* 1997; Lutz, Kukutsch *et al.* 1999). Briefly, $2-4 \times 10^5$ cells were incubated for 30 min with FITC-conjugated dextran (FD40S, Sigma) at 100 $\mu\text{g/ml}$ (a dose selected from preliminary dose-response curves; 50 μg -2 mg/ml). The incubations were either done on ice to control for non-specific binding of the FITC-dextran or at the endocytosis-enabling temperature of 37°C . The cells were then washed with ice-cold phosphate-buffered

saline (PBS), fixed with 100 µl of 1% paraformaldehyde and were analyzed with a FACScan, as above.

4.2.4 Cytokine ELISAs

The basic ELISA protocol employed has been reported previously (Gordon, Zhang *et al.* 2000). For the cytokine assays paired capture and biotinylated detection antibodies and recombinant protein standards were individually optimized for the assays, but otherwise employed as recommended by the supplier. The recombinant cytokine standard curves covered the concentration range of 0–1000 pg/ml; cytokine values are expressed in pg/ml. The BM-DC culture supernatants, BAL and mononuclear cell culture supernatant fluids were not diluted for assay, but plasma samples were diluted 1:10 in PBS-0.5% Tween20 (Polyoxyethylene-sorbitan monolaurate, Sigma Chemical Co.) (PBST).

Immunolon-4 96-well flat-bottom plates (Dynatech laboratories Inc., Chantilly, VA) were coated overnight at 4°C with the appropriate capture antibody in 50 µl of coating buffer (1M NaHCO₃, 1M Na₂CO₃; pH 9.6). The next day, the wells were washed twice with PBST and blocked for two hours at room temperature with 200 µl of PBST supplemented with 10% heat-inactivated (56° C for 1 hour) FBS. Following another 4 washes, 100µl volumes of recombinant protein standards or samples were added to the wells. The recombinant proteins were diluted in the same medium contained within the samples, while blank wells were filled with 100 µl of PBST and plates covered and incubated at 4° C overnight. Biotinylated antibodies (100 µl volumes) were added to the wells after four washes with PBST and the plates were incubated at 37° C for 1 hour. Following another six washes with PBST, 100 µl of streptavidin-conjugated horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA), diluted 1:1000 in PBS/10%

FBS, was added to each well and the plates were again incubated at 37° C for 1 hour. The plates were then washed 8 times with PBST and 100 µl of 2, 2'-azinobis (3-ethylbenthiazoline-sulfonic acid) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. After 5-15 min (when the color developed), the plates were read at an wavelength of 405 nm by a computer-assisted ELISA plate reader (Bio-Rad Model 3550 Microplate reader, Bio-Rad Laboratories, Hercules, CA) utilizing the Microplate Manager program for the Macintosh. The results were expressed in pg/ml (\pm SEM) with reference to the standard curves. All the samples were assayed in duplicate. Capture and biotinylated detection antibody pairs and recombinant protein standards for IFN- γ , IL-1 β , IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, TGF- β , TNF- α and GM-CSF (R & D Systems, Minneapolis, MN) were used.

4.2.5 OVA-specific antibody ELISA

For the OVA-specific IgE assays, the wells were coated with 50 µl of goat anti-mouse IgE capture antibody (2 mg/ml, 1M NaHCO₃, 1M Na₂CO₃, pH 9.6) and the plasma/serum samples were diluted 1:10 in PBST. The washing of the plates was done as before (section 4.2.4). The use of biotinylated OVA at 2 µg/ml (prepared as noted in section 4.6) as the detection reagent for the IgE assay dramatically increased the sensitivity of the assay, but prohibited expression of the data in terms of a absolute concentration of immunoglobulin, so that these results are presented in optical density (OD₄₀₅) units.

The wells of 96-well Immulon-4 plates were coated with 50 µl of 20 µg/ml OVA (IgG1 and IgG2a assays) in coating buffer or capture antibodies in coating buffer (IgE assay) overnight at 4° C. The rest of the protocol performed was identical to the one mentioned in section 4.2.4. The standard curves for IgG1 and IgG2a were generated from recombinant antibody standards

and covered the range of 0-10,000 picograms (pg)/ml. The capture antibodies and recombinant proteins for IgG1, IgG2a, goat anti-mouse IgE capture antibody, and biotinylated anti-mouse IgG1 and IgG2a were purchased from PharMingen Canada Inc.

4.2.6 Chemotaxis assay

The chemotactic responses of the three populations of DCs to MIP-1 α and MIP-3 β (R & D Systems, MN) were examined using modified Boyden microchemotaxis chambers (Neuroprobe, Gaithersburg, MD) with polyvinylpyrrolidone-free 5 μ m pore-size polycarbonate filters. Ten-fold dilutions of the chemokines MIP-1 α and MIP-3 β (0.1 ng/ml to 100 ng/ml) were placed in the lower chambers of duplicate wells, while the DC populations (2×10^6 /ml) were placed in the upper chambers. After incubation for 2 hrs at 37°C the cells that had not migrated into the membranes were wiped from the upper surfaces with a clean glass slide, and then the membranes were fixed and stained using a Diff-Quik kit (American Scientific Products, McGaw Pk, IL). For each sample, the numbers of cells that had migrated into or through to the underside of the filters were enumerated by direct counting of at least six 40x objective fields. The results are expressed as the mean number of cells/40x field (\pm standard error of mean i.e. SEM).

4.3 Animal Sensitization and Challenge

Our lab has fully characterized the model of Ovalbumin (OVA)-induced asthma or asthma-like disease (ALD) (Schneider, Li *et al.* 2001). Briefly, 6-8 week old BALB/c mice from our institutional breeding colony were sensitized with two intraperitoneal injections of 2 μ g of OVA-alum (200 μ l of saline, 1 mg of alum) on days 0 and 14. On days 30, 32, and 34 the animals were exposed in an enclosed flow-through chamber for 20 min to an aerosol of 1% OVA

in sterile saline, generated using an ultrasonic nebulizer (Ultra-Neb 99, Devilbiss Co., Somerset, PA) set at a flow rate of 0.5 liters/min. The aerosolization chamber was comprised of a polypropylene box (30cm X 17cm X 8.5cm) equipped with an input hose for aerosol delivery (mean particle size < 4 μ m) and an output hose to ensure adequate airflow. By day 35 the mice displayed a severe ALD, characterized by 40-60% airway eosinophilia, strong AHR to methacholine (Mch), high-level serum OVA-specific IgE and IgG1 antibodies and pulmonary expression of Th2 cytokines (Schneider, Li *et al.* 2001; Gordon, Li *et al.* 2005). After a two week period, during which the animal's pulmonary inflammatory responses, but not AHR or Th2 reactivity, subsided, the mice (n = 5/group) were surgically anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A small incision was made through the skin over the trachea, and the fascia and musculature over the trachea were bluntly dissected and carefully reflected with forceps. After this, 20 μ l of saline, either alone or containing 1×10^6 DC_{GM-CSF} (immature), DC_{IL-10} (tolerogenic) or DC_{TNF} (mature) was delivered intratracheally into groups of ALD mice (n = 5) using a 30 gauge needle. Normal control mice were similarly given 20 μ l of saline. The cutaneous incisions in the animals were closed with 2-0 nylon suture thread. AHR was measured weekly and the animals were euthanized at 3 weeks, either without aerosol challenge or one day after receiving a 20-minute allergen aerosol challenge, as above. All samples from each animal were stored at -20°C and assayed independently.

4.4 Airway Hyperresponsiveness (AHR)

AHR was assessed in conscious animals by head-out whole body plethysmography (Schneider, Li *et al.* 2001; Gordon, Li *et al.* 2005). Briefly, air was supplied to the head and body compartments of the plethysmograph via a small animal ventilator (Kent Scientific, Litchfield,

CT) and changes in the airflow through the body compartment were monitored using a flow sensor (TRS3300; Kent Scientific) linked via a preamplifier and A/D board (Kent Scientific) to a computer-driven real-time data acquisition/analysis system (DasyLab 5.5; DasyTec USA, Amherst, NH). The head compartment of the plethysmograph was connected to an ultrasonic nebulizer (Ultra-Neb 99) for exposure of the mice to Mch (Sigma) aerosols (mean particle size < 4µm). The bronchoconstriction data were gathered as running 1-sec means of the airflow at the 50% point in the expiratory cycle (Flow @ 50% TVe1; TV = tidal volume, e = expiratory cycle, 1 = 1 sec). This parameter has been demonstrated previously to accurately reflect bronchiolar constriction, as opposed to alveolar constriction or airway occlusion (Vijayaraghavan, Schaper *et al.* 1993; Vijayaraghavan, Schaper *et al.* 1994). Each mouse was sequentially exposed to aerosols of saline alone and then doubling doses of Mch (0.75–25 mg/ml of saline) over ≈15 min.

4.5 Harvesting of Samples from Mice

4.5.1 Serum collection

Serum was collected, only in the experiments where repeated sampling was required. Blood was withdrawn (every 3 weeks) from the tail vein in conscious mice by making a small nick with a scalpel blade. About 0.2 ml of blood was recovered from each animal into Microtainer Brand Serum Separator Tubes (VWR International, Mississauga, ON) which were centrifuged at 10,000 rpm for 5 min. Serum was then collected and aliquoted into 500 µl polypropylene tube, and stored at -80°C until analyzed.

4.5.2 Plasma collection

Blood was collected immediately post-mortem in 1 ml syringes equipped with a 25-gauge needle and containing 100 µl heparin (1000 U/ml, Sigma Chemical Co.) via cardiac puncture or from the vena cava. Typically, 0.8 - 1 ml of blood was recovered from each animal. Duplicate blood smears were made immediately from each mouse, with the remainder of the blood being put in 1.5 ml polypropylene tubes. All samples were processed separately for each mouse. After approximately 1 hour on ice (while the animals were being processed), the tubes were centrifuged at 2500 rpm for 15 min and the supernatant plasma was collected and aliquoted in 500 µl polypropylene tubes and stored at -80°C until analyzed. The blood smears were stained with Wrights-Giemsa solution (Papp, Middleton *et al.* 2000) and differential counts performed on 200 cells per sample and data is expressed as percentage of cells.

4.5.3 Bronchoalveolar lavage (BAL)

To obtain cells from the airways, BAL was performed. Briefly, the mice were euthanized as indicated above and a surgical incision was made over the trachea, which was exposed with a pair of blunt forceps. A small incision was made in the trachea about three tracheal rings from the larynx, and a length of tygon tubing, equipped at one end with a 23-gauge lock needle attached to 3ml syringe was fed into the trachea and ligated in place with cotton thread. The lavage fluid employed was Ca/Mg-free Hanks' Balanced Salt solution (HBSS; Gibco BRL) containing 1% antibiotics/antimycotics (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B in 0.85% saline; Gibco BRL) and 1% FBS. Two BAL washes of 1.5 ml were performed gently to ensure adequate recovery of airway cells; we routinely recovered ≈70-80% of the injected BAL fluid. The BAL samples were then centrifuged

for 10 min at 400xg, and the supernatants were collected, aliquoted and stored at -80°C, while the cells were resuspended in 0.5 ml PBS and counted. Cytocentrifuge preparations of each sample ($\approx 1 \times 10^5$ cells) were stained with Wrights-Giemsa solution and differential cell counts were done (200 cells/slide) for each mouse.

4.5.4 Lung cell suspensions

Lung digestions were carried out to generate single cell suspensions from the lung tissues as described (Lowman, Rees *et al.* 1988). Briefly, the lungs were removed from each mouse, minced mechanically with scalpels and tweezers and put in 6 ml polypropylene tubes containing 5 ml of digestion medium (HBSS/10% FBS containing 0.75 mg/ml Type 4 collagenase and 2 mg/ml hyaluronidase [both Worthington Biochemical Corporation, Freehold, NJ, USA]). The tissues were digested for 1 hour at 37°C, and then further dispersed by repeated gentle aspiration through a 20-gauge needle using a 5 ml syringe. The cells were filtered through four layers of sterile gauze into conical-bottom polypropylene tubes and washed with complete Dulbecco's Modified Essential Medium (DMEM) supplemented with heat-inactivated FBS, 1% antibiotics/antimycotics (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B in 0.85% saline), 1% of 50 mM of 2- mercaptoethanol and 1% of 200 mM L-glutamine. This medium is our standard culture medium and will be referred to hereafter as complete DMEM-10% FBS. The cells were washed twice by centrifugation at 400xg and the viable cell recovery was determined using a hemocytometer and trypan blue dye. The cells were dispensed at 2×10^6 cells/ml into 24-well tissue culture plates. For assessment of cytokine secretion, the cells were cultured for 48 hours at 37°C, without exogenous OVA in experiments in which mice were aerosolized with OVA 48hr before sacrifice and with OVA (50 µg/ml) when

no such aerosolization had been performed. The culture supernatants were harvested using standard procedures, aliquoted, and frozen at -80°C.

4.6 Biotinylation of Ovalbumin

Biotinylation of OVA was done using EZ-Link NHS-PEO₄-Biotinylation kit (Pierce Chemical Co, Rockford, IL). To 6 ml of OVA was added 135 µl of 20mM solution of NHS-PEO₄-Biotin. After 2hr incubation on ice, the reaction mixture was dialyzed against multiple changes of PBS using Zeba Desalt Spin Column, and then this biotinylated OVA was aliquoted and stored at -80°C. We also tested the level of biotinylation using HABA assay and titrated (dilutions ranging 1:250 – 1:3000) the amounts of antibody required for our IgE assay.

4.7 Assessing the long term effects of DC_{IL-10} treatment

The allergic mice were injected intratracheally with 1×10^6 ^{OVA}DC_{IL-10}, ^{OVA}DC_{GM-CSF}, or ^{OVA}DC_{TNF} or saline alone, each in 20 µl of saline, as above. Normal mice treated with saline were used as additional controls. The AHR to methacholine was assessed weekly for 1-4 weeks and then every two weeks until hyperreactivity waned. OVA-specific plasma IgE levels were determined every three weeks through to 32 weeks post-treatment, when the mice were sacrificed and challenged with 1% OVA aerosol and assessed for airway eosinophilia, antibody responses, BAL cytokine levels and dispersed lung cell cytokine secretion.

4.8 Assessing the effect of multiple DC_{IL-10} treatments and approaches

Allergic lung disease was induced in BALB/c mice as indicated above. At two weeks after the last sensitizing dose of allergen exposure, the mice were given either saline or 1×10^6 OVA-presenting DC_{IL-10} intratracheally, intraperitoneally or subcutaneously (n = 4) every second week for a total of four treatments. The animal's AHR was measured after every treatment as well as 7

and 14 days after the last treatment, then the mice were given allergen aerosols (20 minutes, 1% OVA in saline) and harvested two days later. Animal samples for BAL fluid, plasma, lung cultures, blood smears were collected as indicated above.

4.9 *In vitro* assessment of the effects of $^{OVA}DC_{IL-10}$ therapy.

Asthma-like disease was induced in BALB/c mice as noted above (Schneider, Li *et al.* 2001; Gordon, Li *et al.* 2005). Following the last aerosol exposure, the mice were left untreated for a period of 3 wks to allow T central memory (T_{CM}) cells to develop, then their $CD4^{+}$ cells were purified by positive selection using magnetic cell separation (MACS) from a pool of dispersed lung, spleen and lymph node mononuclear cells, as noted (Gordon, Li *et al.* 2005; Zhang, Huang *et al.* 2005). Briefly, the cells collected from lungs (enzymatically treated), spleens and lymph nodes (mechanically with tweezers) were dispersed; the mononuclear cells were purified by density gradient centrifugation using lymphocyte separation medium (LSM; Valeant Pharmaceuticals, CA, USA) and spun for 15 minutes at 400xg. The cells at the interface were collected and washed with PBS and sedimented by centrifugation for 10 minutes at 300xg, then resuspended and enumerated using a hemocytometer and their viability determined by trypan blue dye exclusion (as above). The cells were sedimented at 250xg for 20 minutes at 10°C. They were then resuspended at 10^7 cells/90 μ l of MACS buffer (PBS [pH=7.2], 5 % FBS and 2 mM EDTA; PBS/EDTA) along with 90 μ l of anti-mouse CD4 paramagnetic beads (Miltenyi Biotec, Auburn, CA) and incubated at 6-12°C for 15 minutes. About 1 ml of the MACS buffer was added per 10^7 cells, which were sedimented (300xg for 10 minutes at 4°C) and resuspended at 10^8 cells/0.5 ml of MACS buffer. These were then applied onto large separation (LS) columns (2×10^9 cell maximum capacity; Miltenyi Biotec) which were then

washed three times with MACS buffer (3 ml/ wash) before the column was removed from the MACS magnet. The bound CD4⁺ cells were flushed out of the column with one 5 ml aliquot of MACS buffer, then one more time with 1 ml of the buffer using a column plunger to rapidly and forcibly expel the cells into a sterile tube. The cells were washed in PBS, then with plain DMEM and resuspended at 2x10⁶/ml in complete DMEM/10% FBS. The purity (≈90-94%) of the CD4 cells was confirmed by FACS.

To assess the effects of ^{OVA}DC_{IL-10} treatments on T cells, 2x10⁶ of the purified CD4⁺ T cells were cultured for three days in complete medium with 2x10⁵ OVA-presenting DC_{IL-10} or DC_{GM-CSF} or DC_{TNF}. To implicate specific effector molecules in the cultures, we assessed the effects of adding purified anti-IL-10R (25 µg/ml; HB12538, American Type Culture Collection (ATCC); generously provided by Dr. Henry Tabel) or anti-TGF-β (25 µg/ml; a kind gift from Genentec Inc., South San Francisco, CA) antibodies, recombinant IL-2 (10 U/ml), or the indoleamine 2,3-dioxygenase (IDO) antagonist, 1-methyl tryptophan (1-MT) (200 µM; Sigma) (Gordon, Li *et al.* 2005). The 72 h culture supernatants were analyzed by ELISA for their Th2 cytokine contents.

4.10 *In vivo* assessments of the mechanisms mediating DC_{IL-10}-dependent tolerization of the asthma-like phenotype.

Asthma-phenotype mice were treated with saline or 1x10⁶ OVA-presenting DC_{IL-10} or DC_{TNF} intratracheally, as above (n = 5-7). On days 12, 15, 18, and 21 post-treatment, the mice were injected i.p. with rat anti-IL-10R (5 mg/kg body weight) (Shi, Pan *et al.* 2003), mouse anti-TGF-β (4 mg/kg body weight) (Liu, Hu *et al.* 2003; Neptune, Frischmeyer *et al.* 2003), or equivalent amounts of isotype control antibodies (rat and mouse IgG, respectively) in 200 µl of pyrogen-free saline. Alternately, on day 12 we surgically implanted s.c. 1-MT or placebo slow

release polymer pellets (reported release rate, 10 mg/day; Innovative Research of America, Sarasota, FL) (Sakurai, Zou *et al.* 2002). On day 23, all mice were given 1% OVA aerosols (20 min), then harvested 2 day later for assessment of systemic and pulmonary responses as above.

4.11 RNA purification and quantitative real-time PCR.

Expression of IDO was determined by quantitative RT-PCR. We isolated the RNA from the upper right lung lobes using RNeasy mini kit (Qiagen). Briefly, about 30 mg of lung tissue was harvested from ALD and OVA-presenting DC_{IL-10}-treated mice and about 600 µl of RLT buffer (i.e., guanidine isothiocyanate) was added. The tissue was disrupted and homogenized using a conventional rotor-stator homogenizer (IKA-Labortechnik, Staufen, Germany) until it was fully dispersed (usually 20-40 seconds). The lysate was centrifuged at 8000x g for 3 minutes (all centrifugation steps were done at 20-25°C) and the supernatant was collected and supplemented with 1 volume of 70% ethanol. About 700 µl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at 8000x g. The flow-through was discarded and another 700 µl of Buffer RW1 (contains ethanol) was added to the RNeasy spin column and spun as above to wash the spin column membrane. After again discarding the flow-through, 500 µl of Buffer RPE was added to the RNeasy spin column, which was spun as above, three times. Lastly, the RNeasy spin column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free water was added directly to the spin column membrane and spun as above for 1 min to elute the RNA. The RNA was quantified using a spectrophotometer and purity was checked measuring 260/280 ratio. The RNA was reverse-transcribed to cDNA using a one step RT-PCR kit (Invitrogen) starting with 500 ng of total RNA (pretreated with DNase 1 for 15 min at 25°C) for each 25 µl of PCR reaction. PCR primer pairs were as follows: IDO, forward primer 5'-AGT GTG TGA ATG GTC TGG TCT CTG-3',

reverse primer 5'-ACA TTT GAG GGC TCT TCC GAC T-3'; β -actin, forward primer 5' –AGA GGG AAA TCG TGC GTG AC-3'; reverse primer 5'-CAA TAG TGA TGA CCT GGC CGT – 3'. The PCR was run in a Mx3005P QPCR system (Stratagene) equipped with the MxPro QPCR software package (Version 3), with PCR parameters set at: 50°C for 30 min, 95°C for 10 min, 95°C for 30 sec, 63°C for 30 sec, 72°C for 30 sec, 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec for up to 40 cycles. All IDO mRNA levels were normalized to beta-actin levels. Controls were used to detect the absence of DNA.

4.12 Statistics.

All data were expressed as the mean \pm SEM. Group differences were examined for significance using single factor analysis of variance. For *in vitro* experiments, paired t-tests were used. For AHR, analysis of covariance and a Fisher's LSD *post hoc* test (XL-stat 2007, Addinsoft, New York, USA) was used. Significance was established at $p \leq 0.05$.

5.0 RESULTS

5.1 *In vitro* characterization of tolerogenic dendritic cells

Normal healthy mice between the ages of 8-10 weeks were sacrificed and their bone marrow harvested and pooled. DCs were cultured from bone marrow for 13 days as indicated in the Material and Methods in the presence of GM-CSF either alone (immature; DC_{GM-CSF}), or with either IL-10 (tolerogenic; DC_{IL-10}) or TNF (mature; DC_{TNF}). DC_{GM-CSF} and DC_{IL-10} displayed smaller dendritic or veil-like processes, while cells DC_{TNF} had relatively prominent dendrites or appendages (Figure 5.1).

5.1.1 Dendritic cell surface markers

On FACS analysis, both DC_{GM-CSF} and DC_{IL-10} showed low to moderate levels for costimulatory molecules required for T cell stimulatory responses compared to the mature DC_{TNF}. The DC_{GM-CSF} and DC_{IL-10} expressed significantly lower levels of CD40 and CD54 (ICAM-1) compared to DC_{TNF}. CD80 (B7.1, a ligand for CD28 and CTLA-4) expression levels were similar for all the three DC populations, while CD86 (B7.2, also a ligand for CD28 and CTLA-4) levels were moderately higher in DC_{TNF} population compared to the DC_{IL-10} group ($p=0.057$, Figure 5.1.1A, 5.1.1B). Surface MHC-II expression by DC_{IL-10} was moderate ($29 \pm 6\%$

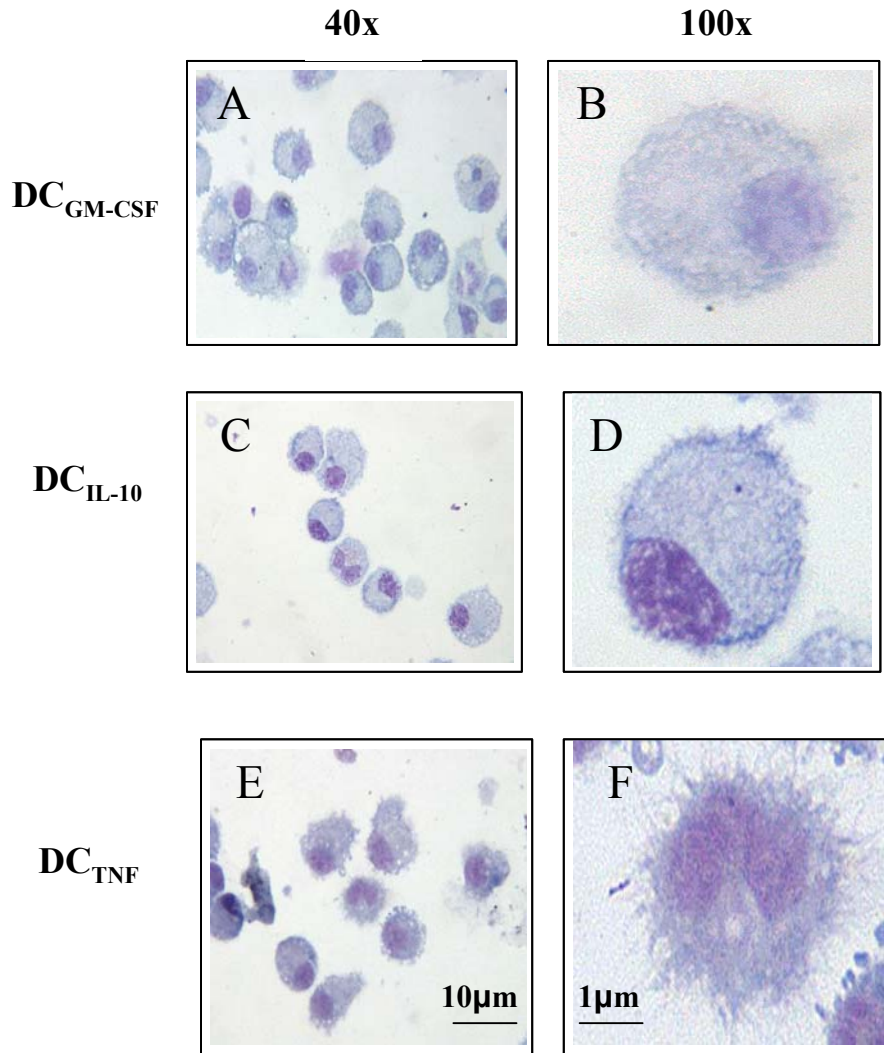


Figure 5.1 Morphological appearance of different populations of dendritic cells following cytocentrifuge preparation. Cytospin slides were prepared for all the three ($^{OVA}DC_{GM-CSF}$ (A and B), $^{OVA}DC_{IL-10}$ (C and D) and $^{OVA}DC_{TNF}$ (E and F) populations of cells using $\approx 1 \times 10^5$ cells from the day 13 cultures and stained with Giemsa-Wright stain. The pictures were taken under the microscope at 40x and 100x magnification.

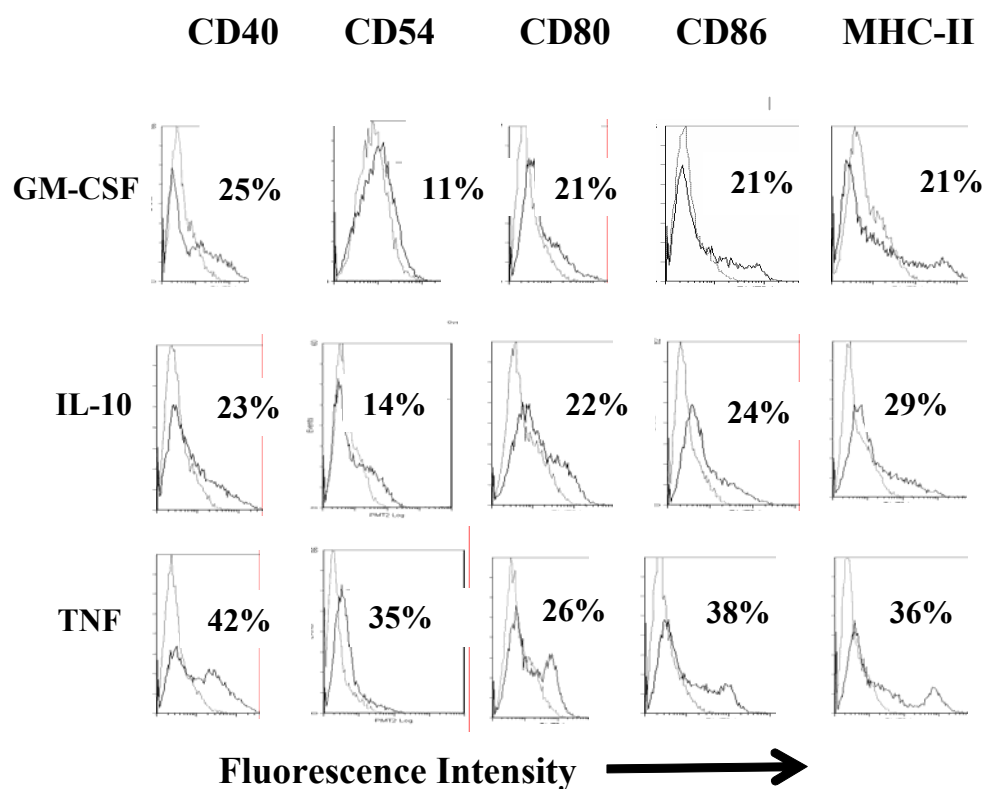


Figure 5.1.1A FACS characterization of immature (DC_{GM-CSF}), tolerogenic (DC_{IL-10}), and mature (DC_{TNF}) bone marrow-derived dendritic cells for a panel of relevant cell surface markers. Bone marrow cells from BALB/c mice were cultured in high (20 ng/ml), then low (7.5 ng/ml) dose GM-CSF and were further treated with GM-CSF alone (immature phenotype), GM-CSF + IL-10 (50 ng/ml) (tolerogenic phenotype) or GM-CSF + TNF- α (10ng/ml) (mature phenotype). On day 13, the cells were stained for analysis of cell surface CD40, CD54, CD80, CD86 and MHC-II by flow cytometry using FITC-labeled antibodies (solid lines). Isotype-matched monoclonal irrelevant specificity antibodies were used as controls (light lines). One representative experiment of five replicates is shown.

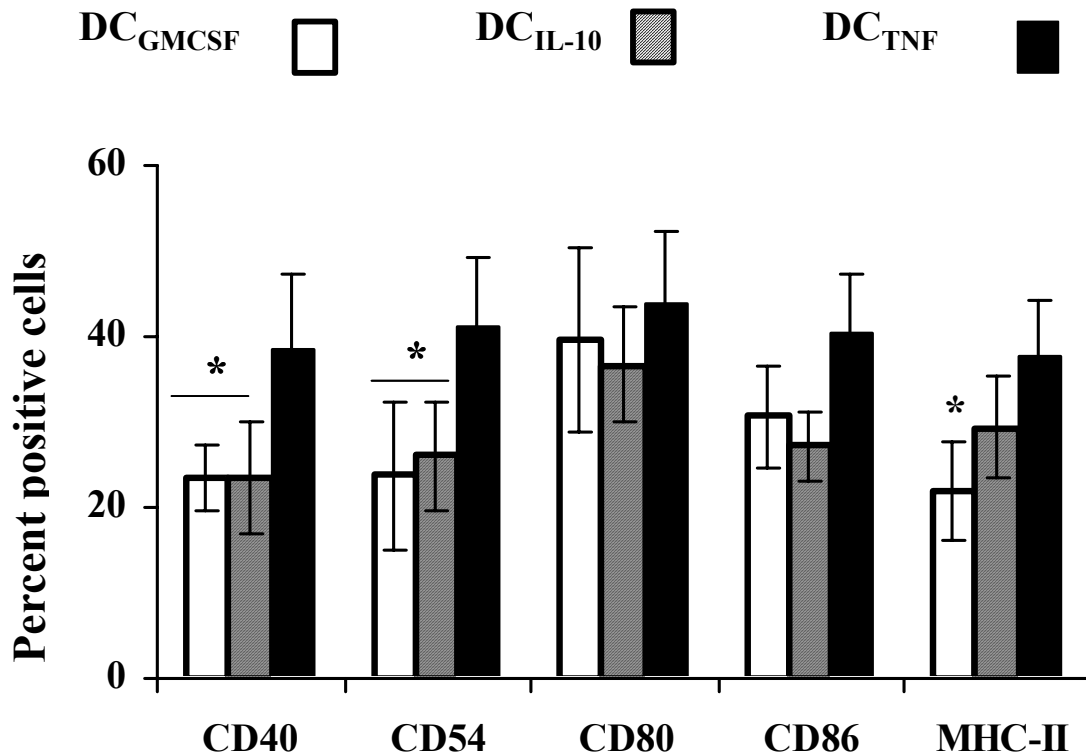


Figure 5.1.1B FACS characterization of immature (DC_{GM-CSF}), tolerogenic (DC_{IL-10}), and mature (DC_{TNF}) bone marrow-derived dendritic cells for a panel of relevant cell surface markers. Bone marrow cells from BALB/c mice were cultured as indicated in Figure 5.1.1A. On day 13, the cells were stained for analysis of cell surface CD40, CD54, CD80, CD86 and MHC-II by flow cytometry using FITC-labeled antibodies. Isotype-matched monoclonal irrelevant specificity antibodies were used as controls (light lines). Mean values \pm SEM from five experiments are shown (This is the similar data as in Figure 5.1.1A). * $p \leq 0.05$ compared to DC_{TNF} group.

of cells were positive, relative to the isotype control) but was not significantly different from the mature populations (Figure 5.1.1A, B). As expected the DC_{GM-CSF} expressed significantly low ($p=0.01$) levels of MHC-II compared to DC_{TNF} population. All three populations expressed moderate levels of CD11b (Mac-1) and CD45RB, and low levels of the other DC markers as CD11c and CD205 (DEC205). The cultured DC populations had moderate levels of expression for markers of monocytes (CD14), macrophages (F4/80) or B cells (CD19) as determined by FACS (Table 5.1.1). The contamination with polymorphonuclear cells was found to be 3 ± 1 % on visual examination of cytopsin slides under the microscope.

To summarize, based on the cell surface marker expression, IL-10 treated-DCs similar to immature DCs (except for MHC-II), expressed low/moderate level expression of costimulatory molecules compared to the mature population.

5.1.2 Functional phagocytic capacity

To assess the capacity of our DCs to endocytose soluble antigens we carried out FITC-dextran (M.W=40,000, similar to our antigen, OVA) phagocytosis assays. The same populations assessed above were incubated with FITC- dextran (50 μ g-2 mg/ml) for 30 minutes at 37°C or 4°C (a control for non- specific FITC-dextran binding). The 100 μ g/ml dose was selected from for subsequent studies. As expected of immature and semi-mature cells (Lutz, Kukutsch *et al.* 1999), DC_{GM-CSF} and DC_{IL-10} avidly phagocytosed FITC-dextran (mean fluorescence intensity [MFI], 6 and 8, respectively; Figure 5.1.2) while DC_{TNF} displayed the very low phagocytic capacity typical of mature cells (MFI, 3).

	CD11b	CD45RB	CD11c	CD205	CD14	CD19	F4/80
GMCSF	29 ±3	24 ±6	11±10	2 ±0	19 ±10	4 ±1	12 ±5
IL-10	45 ±6	18 ±4	15 ±6	3 ±2	20 ±5	8 ±1	19 ±2
TNF	48 ±10	25 ±3	16 ±5	2 ±1	15 ±7	4 ±2	15 ±6

Table 5.1.1 FACS characterization of immature (DC_{GM-CSF}), tolerogenic (DC_{IL-10}), and mature (DC_{TNF}) bone marrow-derived dendritic cells. The indicated DC populations were stained for an array of cell surface markers for alternate cell contaminations. The proportion (expressed as mean % ± SEM) of the cells in each population that were positive for the indicated marker (relative to isotype control antibody staining) are indicated. Data from three experiments are presented.

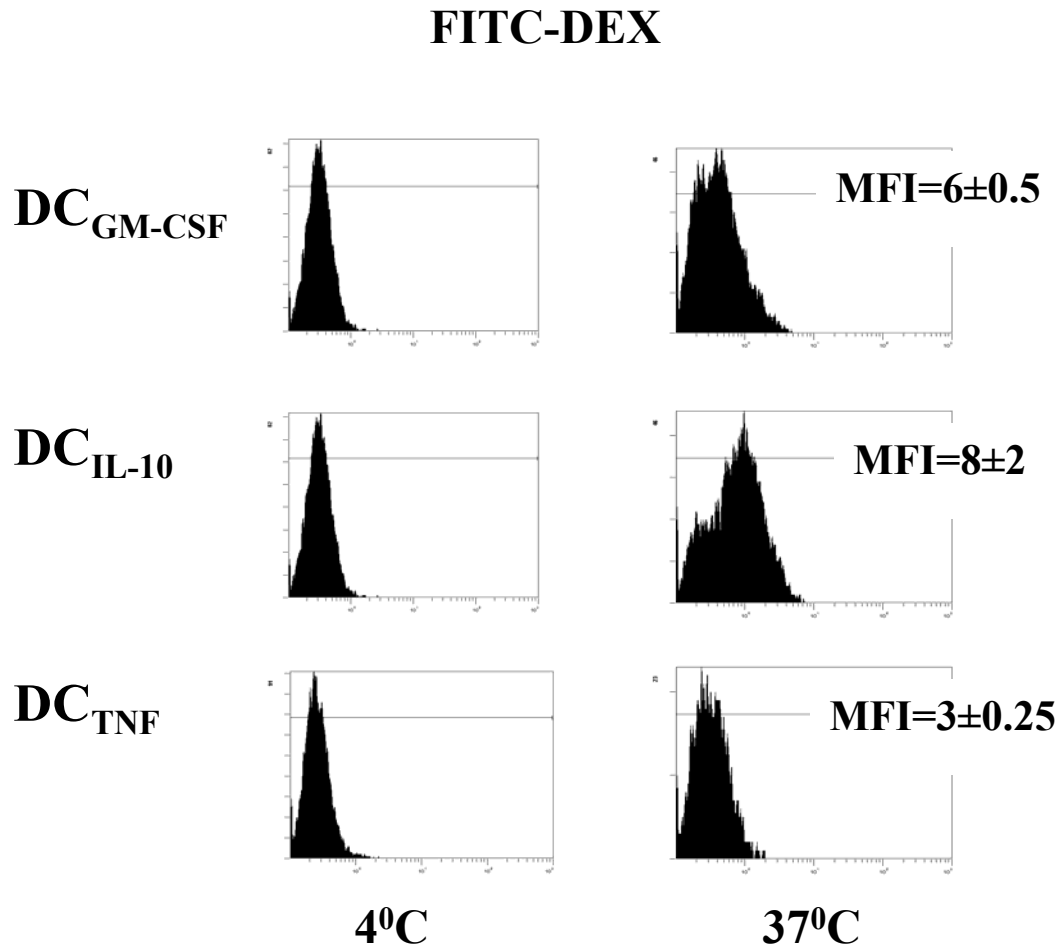


Figure 5.1.2 Phagocytosis of FITC-dextran by immature (DC_{GM-CSF}), tolerogenic (DC_{IL-10}) and mature (DC_{TNF}) generated as in Figure 5.1.1. BM-DC cells were incubated with FITC-dextran (50 µg/ml to 2 mg/ml) on ice or at 37°C for 2 hrs and then washed and assessed by FACS for FITC-dextran uptake. DC_{IL-10} were avidly phagocytic. The graphs on left panel show endocytosis at 4°C. This data is representative of two experiments. Mean fluorescent intensity (MFI) values of 2 experiments are stated.

5.1.3 Capacity of DCs to respond to inflammatory and lymph node-homing chemokines

Immature DCs respond to “inducible chemokines” that are expressed at sites of inflammation (e.g. MIP-1 α [CCL3], a CCR5 ligand). In contrast, mature DCs respond preferentially to chemokines constitutively expressed in T cell-rich areas of secondary lymphoid tissues (e.g. MIP-3 β [CCL19], CCR7 ligand). For this reason, we ran *in vitro* chemotaxis assays with each population of DC, using CCL3 and CCL19 as the agonists, as outlined in section 4.2.5 of Material and Methods. DC_{GM-CSF} and DC_{IL-10} were strongly chemoattracted by CCL3 and only modestly so by CCL19, while DC_{TNF} were CCL19 but not CCL3 responsive (Figure 5.1.3). These above results suggest that the majority of the immature and tolerogenic cells could potentially migrate to inflammatory sites but, at the same time, they showed a modest responsiveness to lymph node-homing chemokines, suggesting that a subpopulation could potentially also migrate to the lung-draining lymph node *in vivo*.

5.1.4 Cytokine secretion

The release of a number of DC-relevant cytokines by our otherwise unstimulated DCs was assessed by ELISA. Specifically, we looked at their expression of immunoregulatory cytokines (IL-10 and TGF- β), inflammatory cytokines (IL-1 β and IL-6) and Th1-skewing cytokines (IL-12 and IFN- γ) as outlined in section 4.2.4 (Figure 5.1.4). DC_{IL-10} secreted significantly higher levels of IL-10 ($p \leq 0.01$) compared to the immature and mature cell

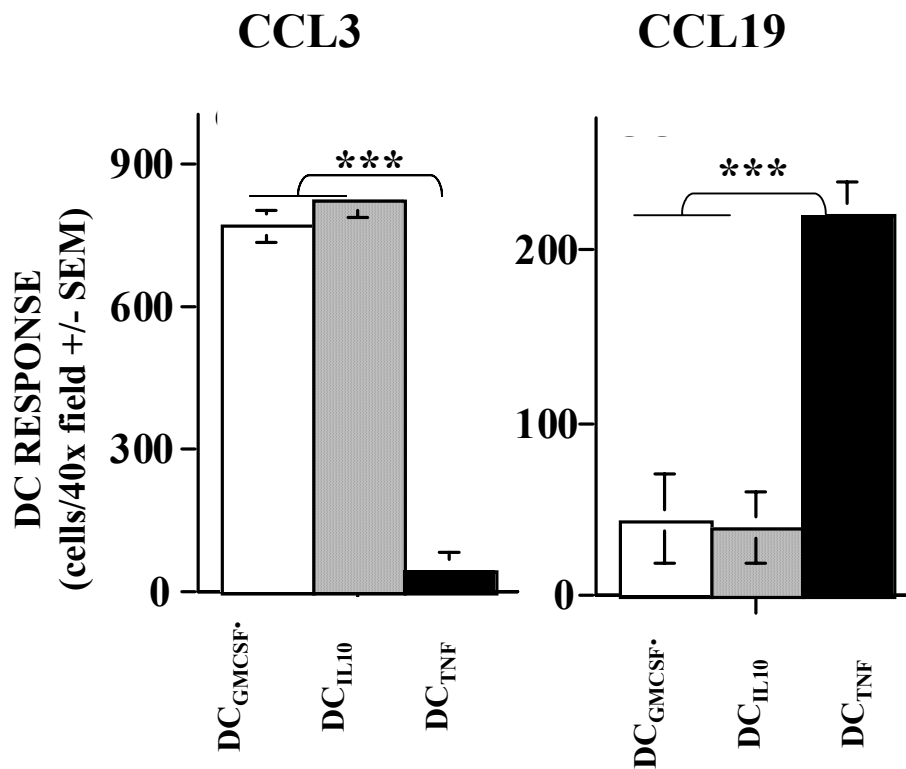


Figure 5.1.3 Chemotaxis assay for BM-DC to CCL3 (left panel) or CCL19 (right panel). The migration patterns of the three populations of DC to chemokines CCL3 (a CCR5 ligand) and CCL19 (a CCR7 ligand) were determined using microchemotaxis assays as indicated in the material and methods. The migration responses at a chemokine concentration of 100 ng/ml are shown above. The dotted line represents the control with no chemokine. The DCs that migrated in response to no chemokine were less than 20 cells for each of three groups of DC. Four experiments with similar results were performed. The results are expressed as mean \pm SEM, *** $p < 0.0001$.

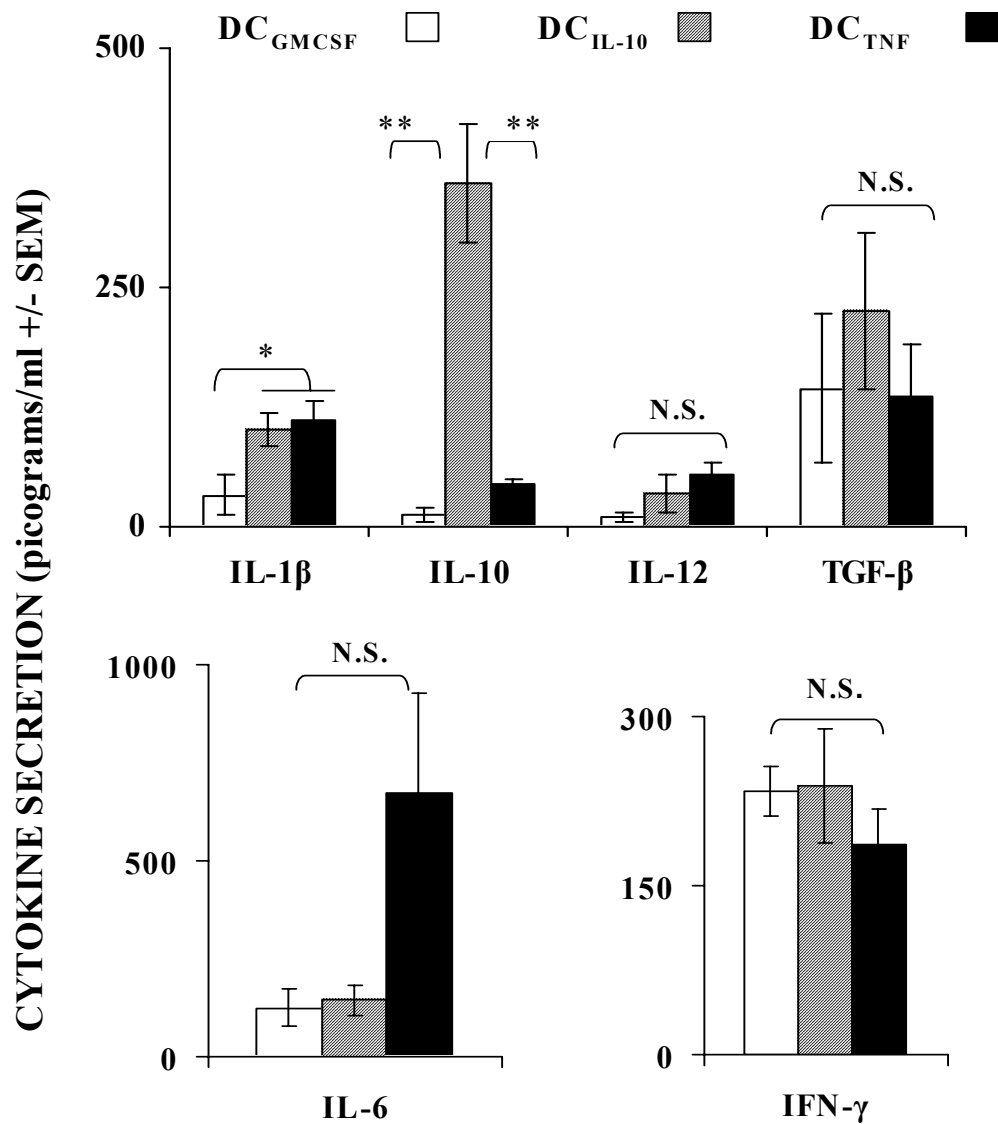


Figure 5.1.4 Differential IL-10 secretion by DC_{IL-10}. All the DC populations from Figure 5.1 (immature, tolerogenic and mature) were cultured for forty-eight hours in RPMI-10 and the supernatants analyzed for IL-1 β , IL-6, IL-10, IL-12, TGF- β and IFN- γ by ELISA, as described in the material and methods. The ELISA was done in duplicate in each experiment; the results depicted represent the mean \pm SEM of five experiments (except for IFN- γ which was assessed only once). * or **, $p \leq 0.05$ or $p \leq 0.01$. N.S. = not significant.

populations. Although, the mean level of TGF- β secretion by DC_{IL-10} was $\approx 40\%$ higher than other two populations, this was not statistically significant ($p \geq 0.05$). Both DC_{IL-10} and DC_{TNF} secreted significantly more IL-1 β ($p \leq 0.05$) than DC_{GM-CSF}, upper panel), and the mean levels of IL-6 in the DC_{TNF} cultures were higher ($\approx 80\%$) than in the DC_{GM-CSF} and DC_{IL-10} cultures, but again this was not statistically significant ($p = 0.06$). Interestingly, there were no differences in IL-12 or IFN- γ expression by the 3 populations.

In conclusion, the IL-10-treated DCs displayed a semi-mature phenotype, expressing low/moderation levels of costimulatory markers. They were avidly phagocytic, migrated in response to inflammatory chemokines and secreted high levels of the immunoregulatory cytokine IL-10. These characteristics would be consistent with IL-10-treated DCs expressing a tolerogenic phenotype.

5.2 *In vivo* tolerization by IL-10-treated dendritic cells

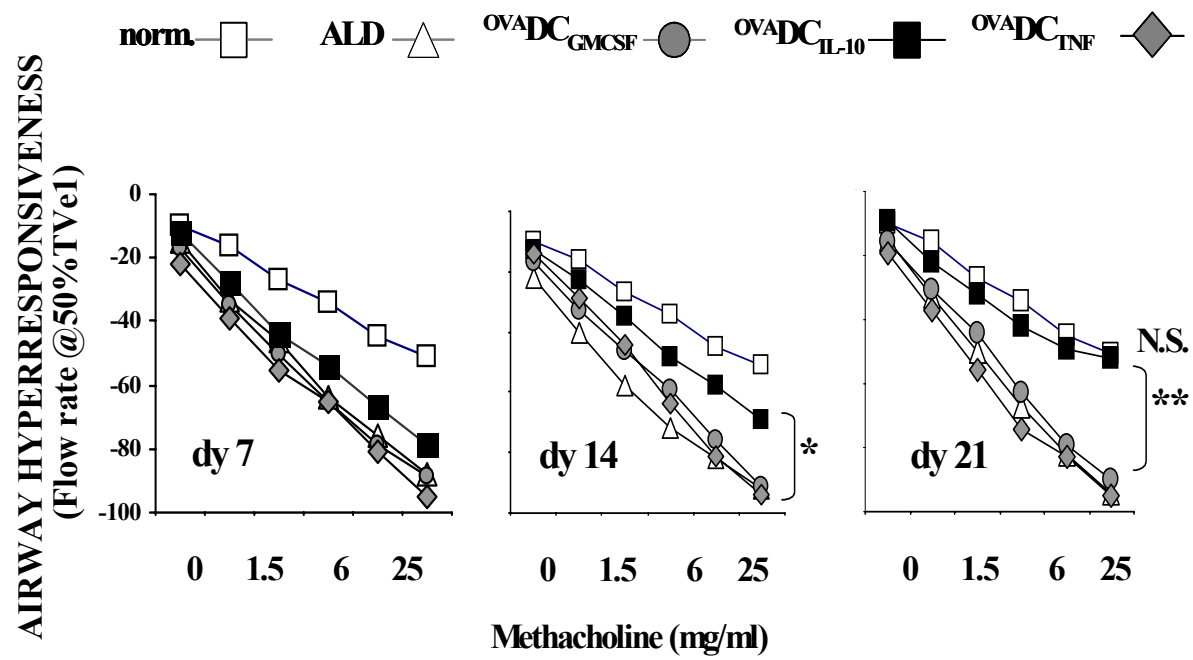
As explained in the material and methods section 4.3, normal healthy BALB/c mice were sensitized with two doses of 2 μg OVA/alum, delivered two weeks apart, and then exposed three times to 1% OVA aerosols (day 30, 32 and 34). They invariably develop many features typical of allergic asthma in humans, including AHR to methacholine, airway eosinophilia (40-70 % eosinophils), and local and systemic Th2-type immune reactivity to allergen (Schneider, Li *et al.* 2001; Gordon, Li *et al.* 2005). In the present study, at two weeks following the last aerosol exposure of the sensitizing regimen, when the allergen-induced eosinophilic inflammatory environment within the airways had waned somewhat (Schneider, 2001; Gordon, 2005), we delivered 1×10^6 OVA-pulsed immature DC_{GM-CSF} (^{OVA}DC_{GM-CSF}), mature DC_{TNF} (^{OVA}DC_{TNF}),

putatively tolerogenic DC_{IL-10} (^{OVA}DC_{IL-10}), or saline (20 µl) alone into the airways of each mouse. We assessed their AHR weekly thereafter, and either exposed them to a 1% OVA aerosol for 20 minutes 48 hrs before sacrifice (day 23), or left them unexposed.

5.2.1 Airway Hyperresponsiveness (AHR)

AHR is the hallmark of asthma and can be measured by several methods. We used a whole-body head-out plethysmograph, which measures the bronchiolar constriction response, as explained in section 4.4. AHR was assessed every week and each mouse was exposed to increasing doses of Mch (0-25 mg/ml) over 15-20 minutes and each mouse was exposed to increasing doses of Mch (0-25 mg/ml) over 15-20 minutes. The air flow rate at the 50% point of the expiratory cycle was measured, and the data expressed as loss of function (i.e., negative values Figure 5.2.1). None of the treatments had significant effects on the AHR of the asthma-phenotype mice when assessed at the one-week time-point, but by day 14 the ^{OVA}DC_{IL-10} group began to show significant (p=0.03) signs of improvement in AHR versus saline-treated asthma-phenotype mice. By day 21 the bronchial responsiveness of the ^{OVA}DC_{IL-10} treatment group was fully normalized, while the ^{OVA}DC_{GM-CSF} and ^{OVA}DC_{TNF} groups as well as the saline-treated animals remained hyperresponsive (Figure 5.2.1; p<0.01, versus saline-treated asthma-phenotype mice). At 3 weeks no significant differences were observed between the normal and ^{OVA}DC_{IL-10}-treated animals. In all experiments in which we assessed AHR (n=15), it was fully normalized within 3 weeks of ^{OVA}DC_{IL-10}-treatment.

Figure 5.2.1 Treatment of asthma phenotype mice with $^{OVA}DC_{IL-10}$ reverses airway hyperresponsiveness (AHR) within 3 weeks. BALB/c mice with severe ALD ($\approx 60\%$ airway eosinophils on airway allergen challenge) were given 1×10^6 $^{OVA}DC_{TNF}$, $^{OVA}DC_{GM-CSF}$, $^{OVA}DC_{IL-10}$ or saline intratracheally. Over the next 3 weeks their AHR was assessed weekly by head-out whole body plethysmography, following exposure to increasing doses of methacholine, as explained in the materials and methods. The data are expressed as running 1-sec means of the airflow rate at 50% point (i.e., 50% tidal volume) in the expiratory cycle. Normal mice were included as negative controls. This experiment is representative of 15 experiments ($n=5$ /group) in which we found that, beginning at 14 days post-transfer, the AHR of $^{OVA}DC_{IL-10}$ -treated mice, but not those treated with either $^{OVA}DC_{TNF}$, $^{OVA}DC_{GM-CSF}$ or saline, disappears. The data represent the mean percent decreases in airflow (i.e., bronchoconstriction) with increasing doses of Mch. The SEM values ranged between 1-3%, but are not presented in order to maintain graphic clarity. The $^{OVA}DC_{IL-10}$ -treated mice were significantly less hyperresponsive than $^{OVA}DC_{TNF}$, $^{OVA}DC_{GM-CSF}$ and saline-treated mice at 2 and 3 weeks post-treatment. * and **, $p \leq 0.05$ and 0.01 .



5.2.2 Airway Eosinophilia

The $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatments also affected eosinophilic inflammation and the Th2 responses of the asthma phenotype mice. In a first series of experiments, saline, $\text{OVA}^{\text{DC}}_{\text{TNF}}$, $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$, or $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ were introduced into the airways two weeks after the last sensitizing regime OVA aerosol exposure, as above, but these mice were not exposed to allergen again. Three weeks later, when the saline-treated ALD mice were still hyperresponsive, the airway eosinophilia of these mice (which by this time had not been exposed to OVA for 5 weeks) had largely waned. On the other hand, the airways of the mice given immunocompetent $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ or $\text{OVA}^{\text{DC}}_{\text{TNF}}$ as antigen-presenting cells showed eosinophilia ($p \leq 0.01$ and $p < 0.05$, versus $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ group), while those of the mice that received the putatively tolerogenic $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ contained fewer eosinophils than the saline-treated ALD controls ($p = 0.02$, Figure 5.2.2A). This also strongly suggests that these immature $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ matured into fully competent APCs *in vivo*.

In our next and all subsequent experiments, the mice were treated with $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$, $\text{OVA}^{\text{DC}}_{\text{TNF}}$, or $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ as above, but they were challenged with OVA aerosols (20 min of nebulized OVA; 10 mg/ml) 48 hrs before sacrifice in order to induce the recall responses. Here we found that the airways of the $\text{OVA}^{\text{DC}}_{\text{TNF}}$, $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ - and saline-treated, asthma-phenotype animals were replete with eosinophils (Figure 5.2.2B), while the eosinophilia of the ALD mice treated 3 wk previously with $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ was reduced by $\approx 59\%$ ($p < 0.01$) relative to the $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ -treated mice, by $\approx 42\%$ ($p = 0.048$) relative to the $\text{OVA}^{\text{DC}}_{\text{TNF}}$ -treated mice and by 51% ($p = 0.02$) relative to the saline-treated ALD mice. Thus, $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatment of asthma phenotype mice ameliorates eosinophil influx into the airways following allergen challenge, one of the most pathogenic components of the late phase asthmatic response (Flood-Page, Menzies-

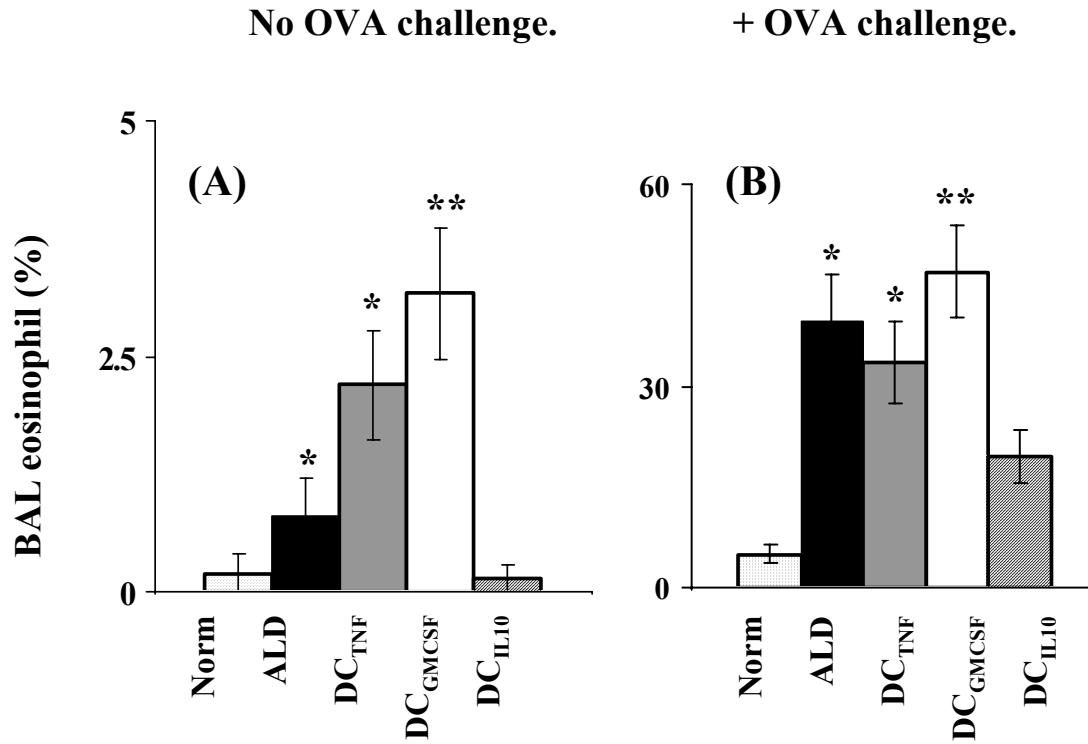


Figure 5.2.2 ^{OVA}DC_{IL-10}-treatments diminish airway eosinophilia following allergen recall response challenge. BALB/c mice with severe ALD were treated as in Figure 5.2.1 and were harvested after 3 weeks without (A) or 48 hrs after (B) aerosol challenge with 1% OVA aerosol (20 min exposure). The airway cells were recovered by bronchoalveolar lavage and differentials done on 200 cells from each mouse. The controls were the saline-treated ALD mice, ^{OVA}DC_{TNF}, ^{OVA}DC_{GM-CSF} and normal mice. * or ** = $p \leq 0.05$ or $p \leq 0.01$, respectively versus the ^{OVA}DC_{IL-10} group. This is representative of four experiments. Mean values \pm SEM of five mice per group are presented.

Gow *et al.* 2003).

5.2.3 Antibody Responses

We also examined the effects of the treatments on the levels of plasma OVA-specific antibodies in the treated animals. Aerosolization of the mice 48 hr before sacrifice had no discernible effect on the circulating OVA-specific antibody levels relative to non-aerosolized mice. So, the data presented here is representative of all the five +/- aerosol experiments. We observed significant reductions in IgE levels following $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ treatment relative to the saline-treated ALD mice in all 5 of 5 experiments (Figure 5.2.3A). In 4 experiments depicted, no differences were detected in IgE levels in $\text{OVA}^{\text{A}}\text{DC}_{\text{GM-CSF}}$ - or $\text{OVA}^{\text{A}}\text{DC}_{\text{TNF}}$ -treated mice relative to the $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ -treated mice at 3-week time point. However, in one other experiment, the $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ -treated mice had 35% lower IgE levels than $\text{OVA}^{\text{A}}\text{DC}_{\text{GM-CSF}}$ -treated animals ($p \leq 0.05$) and in 30% lower IgE than $\text{OVA}^{\text{A}}\text{DC}_{\text{TNF}}$ -treated mice ($p \leq 0.05$). Overall, the OVA-specific plasma IgE levels were not reduced within DC groups 3 wks following $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ treatment.

Interestingly, in four experiments, the circulating levels of OVA-specific IgG1 were significantly reduced in the $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ - relative to saline-treated ALD mice ($p \leq 0.01$; Figure 5.2.3B). In three of these OVA-specific IgG1 levels were significantly lower ($p \leq 0.05$) in the $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ -treated mice relative to $\text{OVA}^{\text{A}}\text{DC}_{\text{TNF}}$ -treated animals (Figure 5.2.3B), while no differences were detected between $\text{OVA}^{\text{A}}\text{DC}_{\text{GM-CSF}}$ - and $\text{OVA}^{\text{A}}\text{DC}_{\text{IL-10}}$ -treated mice.

We also assessed the circulating levels of OVA-specific IgG2a, to determine whether our treatment success was attributable to immune deviation (i.e. $\text{Th2} \rightarrow \text{Th1}$). We observed no

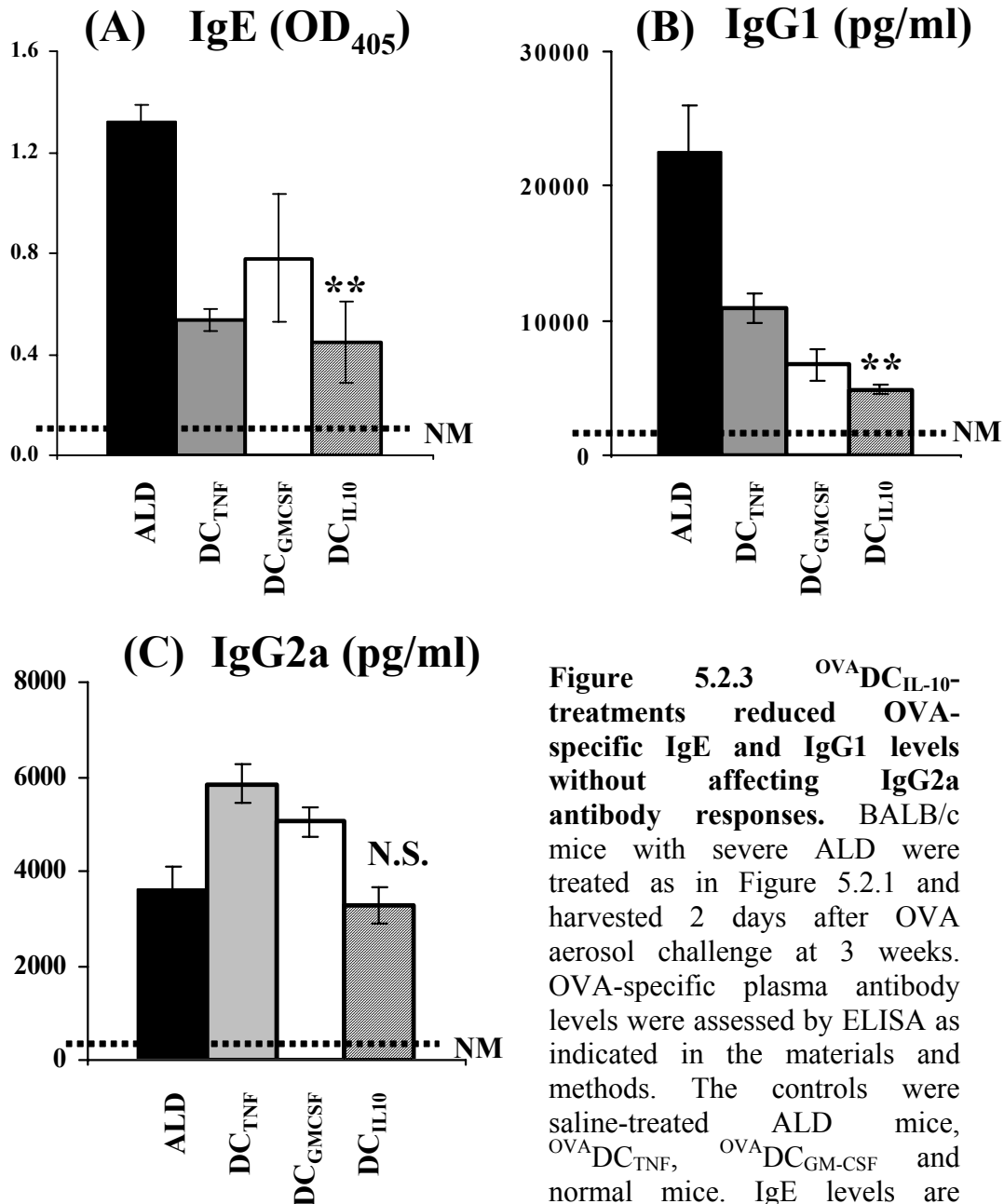


Figure 5.2.3 ^{OVA}DC_{IL-10}-treatments reduced OVA-specific IgE and IgG1 levels without affecting IgG2a antibody responses. BALB/c mice with severe ALD were treated as in Figure 5.2.1 and harvested 2 days after OVA aerosol challenge at 3 weeks. OVA-specific plasma antibody levels were assessed by ELISA as indicated in the materials and methods. The controls were saline-treated ALD mice, ^{OVA}DC_{TNF}, ^{OVA}DC_{GM-CSF} and normal mice. IgE levels are expressed as mean OD₄₀₅ ± SEM

IgG1 and IgG2A are expressed as mean ± SEM (5 mice/group) in pg/ml and the data is representative of 4 experiments each. All comparisons were done relative to ALD mice. ** = $p \leq 0.01$ and N.S. = non-significant. The dotted line in each graph represents the values in normal mice.

significant differences in plasma IgG2a levels between $^{OVA}DC_{IL-10}$ -treated and saline-treated asthma phenotype mice (Figure 5.2.3C). In summary, $^{OVA}DC_{IL-10}$ treatment significantly reduced both the OVA-specific IgE and OVA-specific IgG1 antibody responses while the levels of IgG2a antibody remained unaffected.

5.2.4 Impact of $^{OVA}DC_{IL-10}$ treatment on airway cytokine levels

We also assessed the pulmonary Th2 cytokine responses of the saline-, $^{OVA}DC_{TNF}$ -, $^{OVA}DC_{GM-CSF}$ - and $^{OVA}DC_{IL-10}$ -treated ALD animals. As above, in the first series of experiments, mice treated 3 wk earlier with saline, $^{OVA}DC_{TNF}$, $^{OVA}DC_{IL-10}$ and $^{OVA}DC_{GM-CSF}$ were euthanized without further exposure to antigen. Bronchoalveolar lavage (BAL) was done on each mouse (n=5/group) and the BAL fluid was tested for IL-4, IL-5, IL-9, IL-12 and IL-13 (Figure 5.2.4A). The $^{OVA}DC_{IL-10}$ -treated mice showed no significant decreases in the levels of IL-4, -5, -9 and IL-13 versus saline-treated ALD mice (Figure 5.2.4A, upper panel). In addition, no difference in cytokine levels, except IL-5 (p=0.01), was seen in the $^{OVA}DC_{IL-10}$ -treated group compared to the $^{OVA}DC_{GM-CSF}$ -treated group. $^{OVA}DC_{TNF}$ -treated animals also displayed no differences in BAL cytokines versus $^{OVA}DC_{IL-10}$ -treated mice. The expression of IL-12 was significantly depressed (80-88%, p=0.01) in the $^{OVA}DC_{IL-10}$ -treated animals relative to both the $^{OVA}DC_{TNF}$ - and $^{OVA}DC_{GM-CSF}$ -treated mice (Figure 5.2.4A, lower right panel) but no significant differences were detected relative to saline-treated ALD mice.

In a second series of experiments, we exposed the mice to allergen aerosols 48 hrs before sacrifice and found significantly reduced cytokine levels in the BAL of the $^{OVA}DC_{IL-10}$ -treated mice compared to the saline-treated animals. Thus, one allergen exposure served as a full recall

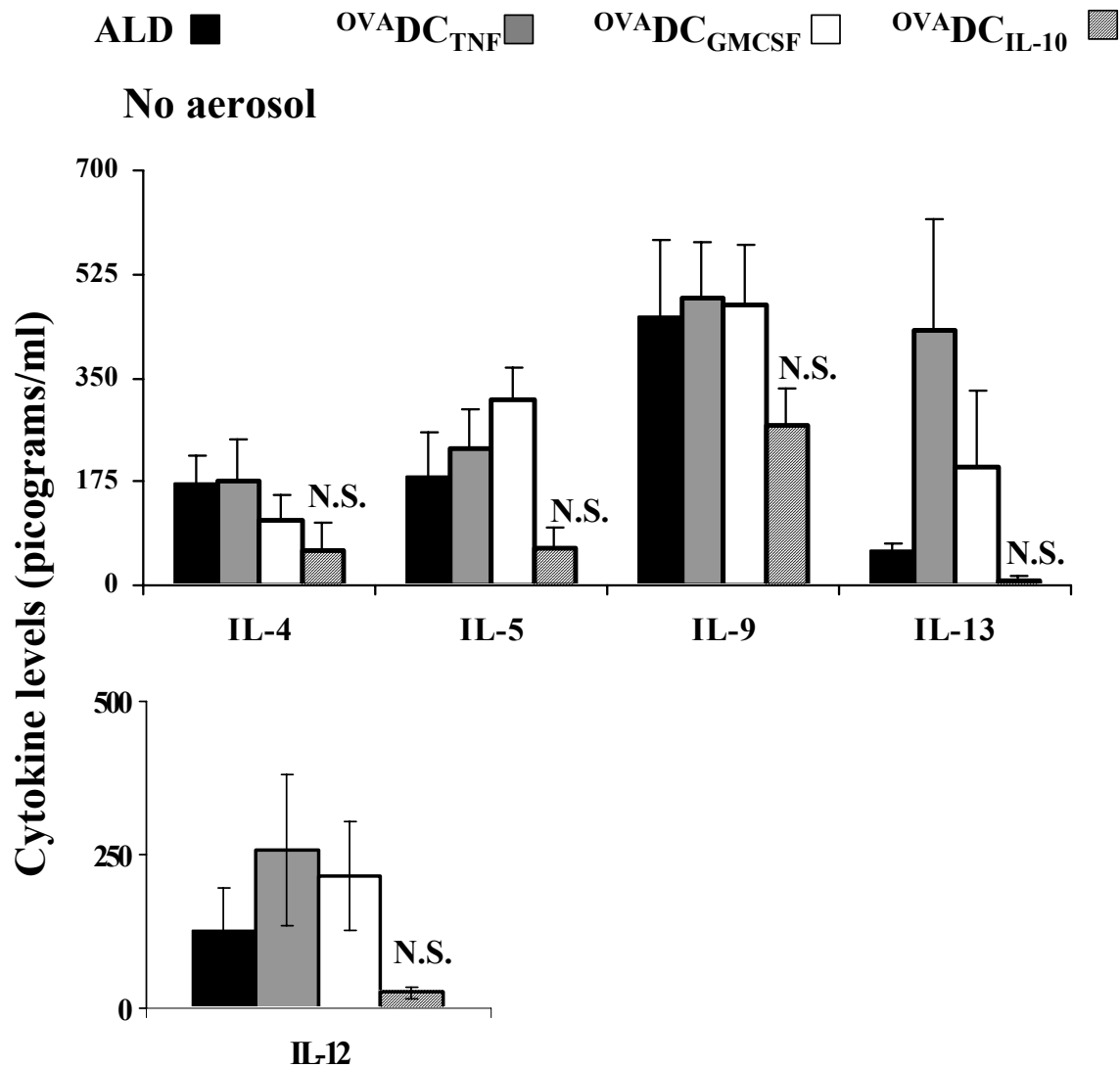


Figure 5.2.4A Effect of $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatments on BAL fluid cytokine levels. BALB/c mice with severe ALD were treated as in Figure 5.2.2 and 5.2.3 and were harvested after 3 weeks without prior OVA re-challenge and assessed for cytokine levels in the BAL fluid by ELISA as stated in the materials and methods. The data is representative of 2 experiments (n=5/group). Data was normalized using log transformations and statistics was done on log-transformed data. N.S. = not significant versus ALD saline controls.

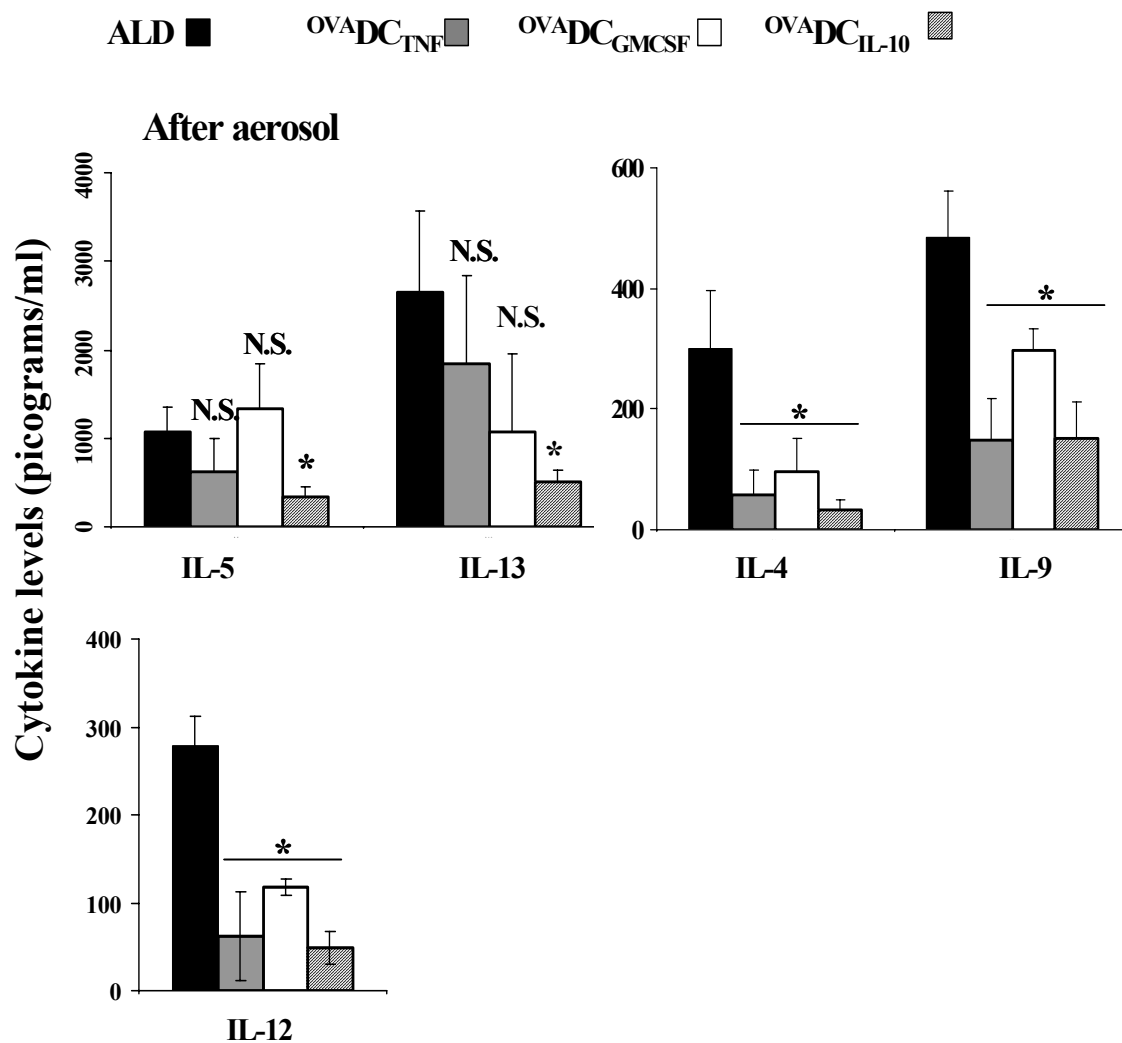


Figure 5.2.4B Effect of OVA^ADC_{IL-10} treatments on BAL fluid cytokine levels. BALB/c mice with severe ALD were treated as in Fig 5.2.2 and 5.2.3 and were harvested, 48 hrs after OVA challenge (day 23), and assessed for cytokine levels in the BAL fluid by ELISA as stated in the materials and methods. The data is presented as means \pm SEM of 4 experiments (n=4-5/group). * $p \leq 0.05$ versus ALD-saline control. N.S. = not significant versus saline-treated ALD mice.

challenge, and reverted the ALD mice back to a complete asthma phenotype (i.e., AHR, eosinophilia and Th2 cytokines, Figure 5.2.4B). All the cytokines i.e. IL-4, -5, -9 and -13 were significantly reduced ($p \leq 0.01$ for IL-4, IL-9 and IL-13, $p \leq 0.05$ for IL-5) in $^{OVA}DC_{IL-10}$ -treated animals relative to saline-treated ALD mice (Figure 5.2.4B). Marked differences were also detected between these two groups with respect to the levels of IL-12 ($p \leq 0.05$). However, exposure to aerosol partially affected the BAL cytokine profile within the $^{OVA}DC_{TNF-}$, $^{OVA}DC_{GM-CSF-}$ and $^{OVA}DC_{IL-10}$ -treated groups. $^{OVA}DC_{GM-CSF}$ -treated animals secreted significantly increased BAL IL-5 ($p=0.04$) versus $^{OVA}DC_{IL-10}$ -treated animals. BAL IL-5 and -13 levels were not significantly different in $^{OVA}DC_{GM-CSF-}$ or $^{OVA}DC_{TNF-}$ treated animals (Figure 5.2.4B, upper panel first graph) compared to saline-treated ALD controls. Interestingly, like $^{OVA}DC_{IL-10}$ -treated animals we found that IL-4 and -9 levels in $^{OVA}DC_{GM-CSF}$ group as well as in $^{OVA}DC_{TNF}$ group were also significantly ($p \leq 0.05$) lower compared to the ALD mice.

The above observations show that the $^{OVA}DC_{IL-10}$ treatment of allergic mice reduced all the BAL Th2 cytokines without affecting the expression of Th1- skewing cytokines, while $^{OVA}DC_{TNF}$ and $^{OVA}DC_{GM-CSF}$ were also able to partially ameliorate the Th2 responses.

5.2.5 Pulmonary parenchymal responses

Following BAL, the lungs were removed from each animal, and single cell suspensions were prepared by enzymatic dispersion of the tissues (see section 4.5.4). The cells were enumerated and plated at 2×10^6 cells/ml with or without OVA for 48 hours at $37^\circ C$. The lung culture supernatants were harvested after 2 days and analyzed for various cytokines by ELISA.

Since, we thought that exposure to OVA aerosol might elicit the movement of the cells to the airways, we performed two series of experiments. In the first line of experiments, we harvested the mice without aerosol allergen challenge 48 hrs earlier and cultured the lung mononuclear cells with OVA (50 µg/ml). There was no appreciable difference in the cytokine secretion of saline- and $^{OVA}DC_{IL-10}$ -treated animals. This might be due to the difference in the DC: T cell ratios in the ALD whole lung cultures compared to other cultures from DC-treated groups. Since very low amount of cells were obtained on purification of single lung cultures so we did not titrate the amount of DCs to T cells. Also, we observed that IL-4, IL-9 and IL-13, but not IL-5 secretion was significantly lowered ($p \leq 0.05$) in $^{OVA}DC_{IL-10}$ -treated group relative to the $^{OVA}DC_{GM-CSF}$ -treated group (Figure 5.2.5A). In addition, only IL-5 secretion was markedly lowered ($p \leq 0.05$) in $^{OVA}DC_{IL-10}$ -treated mice relative to $^{OVA}DC_{TNF}$ -treated mice. There was no difference in IL-12 secretion within any of the groups.

In a second series of experiments, we aerosolized the mice with 1% OVA 3 weeks post-treatment and sacrificed them two days later. We observed no significant differences in the secretion of Th2 cytokines between saline and $^{OVA}DC_{IL-10}$ treatments (Figure 5.2.5B). In addition, we observed that IL-5 secretion was significantly lowered ($p \leq 0.05$) in $^{OVA}DC_{IL-10}$ -treated animals relative to $^{OVA}DC_{GM-CSF}$ - and $^{OVA}DC_{TNF}$ -treated animals (Figure 5.2.5B) while IL-13 levels were decreased compared to $^{OVA}DC_{TNF}$ -treated mice.

The parenchymal responses above depicted a different picture than the BAL fluid responses. As no differences were detected between the saline-treated ALD and $^{OVA}DC_{IL-10}$ -treated mice, this suggests that the airway compartment responds separately from the parenchymal compartment, at least in the terms of kinetics or that the tolerance has yet not been achieved in the latter. Besides this, the DC:T cell ratio in the cultures may also cause this

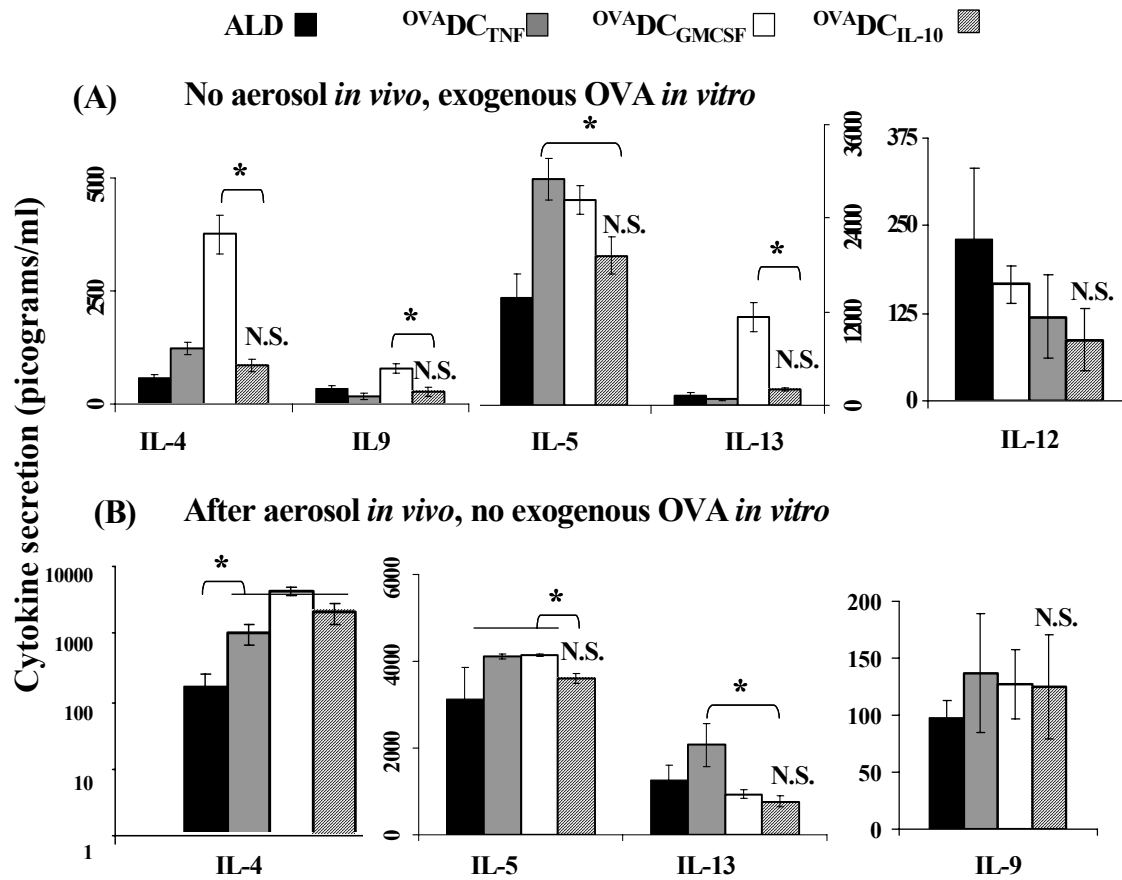


Figure 5.2.5 Effect of OVA⁺DC_{IL-10} treatments on lung parenchymal mononuclear cell cytokine secretion. BALB/c mice with ALD were treated as above and were harvested after 3 weeks with (A) or without (B) OVA challenge. The lung mononuclear cells were purified from enzyme-dispersed lung tissues (section 4.5.4, materials and methods), then established in culture @ 2×10^6 cells/ml, with (A) or without (B) exogenous OVA (50 μ g/ml) for 48 hrs. The culture supernatants were assessed for the indicated Th2 cytokines by ELISA. The upper and the lower panels are representative of 2 experiments each. N.S. = not significant versus the saline-treated ALD animals. * $p \leq 0.05$.

difference as the whole lung cultures from the mice treated in a similar manner with different DC populations showed significant differences. Immunoreactive $^{OVA}DC_{GM-CSF}$ significantly enhanced the allergic responses (IL-4, -9 and -13, $p \leq 0.05$) relative to $^{OVA}DC_{IL-10}$ -treated mice (Figure 5.2.5A) on exogenous OVA addition in cultures while the ALD mice which had not received 10^6 DCs earlier showed diminished responses. However, only IL-5 levels were decreased in $^{OVA}DC_{IL-10}$ -treated animals (Figure 5.2.5B) suggesting that tolerance might just be starting to initiate in this compartment and IL-5 might be the first cytokine to be affected in this phenomenon.

To summarize the above *in vivo* observations, $^{OVA}DC_{IL-10}$ -treated animals show physiologically normal airway function 3 weeks post-treatment. At this point, in time if they had not been exposed to recall antigen challenge *in vivo* they show overall reduced eosinophilia and OVA-specific IgG1, with no discernible difference in Th2 cytokine secretion in the BAL or by lung parenchymal mononuclear cells relative to saline-treated ALD mice. The antigen challenge of mice at this time point revealed that there still existed a potential to develop eosinophilia. The OVA-specific IgE and IgG1 antibody levels were reduced moderately but not to the background levels. The BAL fluid cytokine profile showed significantly reduced levels compared to saline-treated ALD controls but not $^{OVA}DC_{GM-CSF}$ or $^{OVA}DC_{TNF}$. The parenchymal Th2 responses of the $^{OVA}DC_{IL-10}$ -treated animals were not different from ALD mice. Since IL-5 cytokine levels were significantly reduced in $^{OVA}DC_{IL-10}$ -treated mice versus other DC groups suggests that tolerance induction had occurred in the bronchoalveolar component and was beginning to initiate in lung parenchyma and IL-5 may be the first cytokine to be effected (discussed later).

5.3 Time period for effectiveness of DC_{IL-10} therapy

To determine whether the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ therapy could induce robust tolerization of the asthmatic responses given sufficient time, we next treated the mice as above with $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ and followed the tolerization process across an 8 month period. We monitored AHR and serum IgE levels as a non-invasive surrogate measure of tolerance. The ALD mice were given saline, $\text{OVA}^{\text{DC}}_{\text{TNF}}$, $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ or $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ intratracheally, and then we assessed levels of OVA-specific-IgE antibody levels every three weeks until they were near background levels. We then aerosolized the mice at 32 weeks, two days before harvest and assessed the asthmatic phenotype of our mice as noted above.

5.3.1 Airway hyperresponsiveness (AHR)

AHR was measured weekly for 4 weeks from the time DCs were injected into the airways, then every second week until 10 weeks, and finally every third week until 16 weeks. Interestingly, the tolerogenic effects of the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatment on the animals' AHR were transient. AHR was completely normalized at 3 weeks and at 2 months post-treatment (Figure 5.3.1) ($p \leq 0.01$; relative to saline-, $\text{DC}_{\text{GM-CSF}}$ - or $\text{OVA}^{\text{DC}}_{\text{TNF}}$ -treated ALD mice). At 10 weeks the bronchial responsiveness of the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ group was still significantly different from the $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ -treated animals ($p \leq 0.01$), but hyperresponsiveness had begun to return, such that this group was now also significantly different from the normal controls ($p = 0.024$). The AHR was only borderline in the $\text{OVA}^{\text{DC}}_{\text{TNF}}$ - and saline-treated ALD animals ($p = 0.06$ and $p = 0.055$ respectively) versus the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated animals. Moreover, at 13 weeks post-treatment the AHR of the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated group had fully reverted to the asthma phenotype ($p > 0.05$). Thus, the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatment only transiently (for ≈ 10 weeks) normalized airways function.

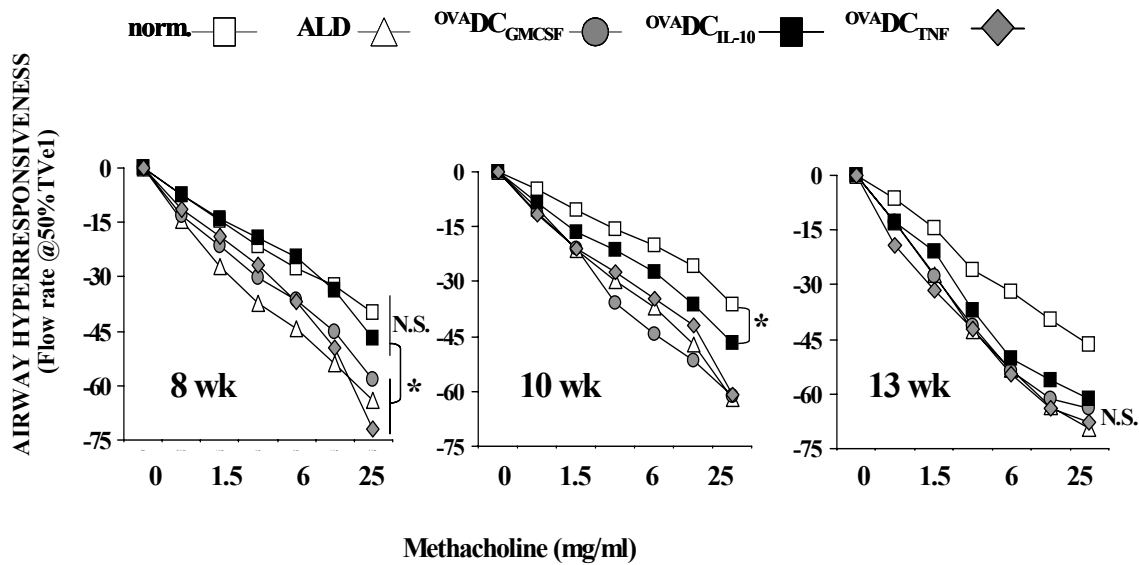


Figure 5.3.1 $\text{OVA DC}_{\text{IL-10}}$ -dependent amelioration of AHR is transient. BALB/c mice with severe ALD were given 1×10^6 $\text{OVA DC}_{\text{IL-10}}$, $\text{OVA DC}_{\text{GM-CSF}}$, $\text{OVA DC}_{\text{TNF}}$ or saline intratracheally as above and AHR was measured over 13 weeks using head-out whole body plethysmography with increasing doses of methacholine (0 to 25 mg) as explained in the materials and methods. The data depict AHR of the mice at 8, 10 and 13 weeks post-treatment as in Figure 5.2.1. This experiment is representative of 3 others performed. Each data point represents the mean value of 5 mice. The SEM values ranged between 1-5% (at 8wks), 2-6% (at 10 wks) and 1-4% (at 13 wks). The $\text{OVA DC}_{\text{IL-10}}$ -treated mice were significantly ($p \leq 0.01$) different from $\text{DC}_{\text{GM-CSF}}$ - or saline-treated mice at 8 and 10 weeks post-treatment, and were significantly different from DC_{TNF} only until the 8 week time point. * $p \leq 0.05$.

5.3.2 Airway Eosinophilia

As noted above, the ALD mice that had been treated 32 weeks earlier with saline, $^{OVA}DC_{TNF}$, $^{OVA}DC_{GM-CSF}$ or $^{OVA}DC_{IL-10}$ were exposed for 20 minutes to OVA aerosols and sacrificed 48 hrs later. We found that the airways of the mice given immunocompetent $^{OVA}DC_{GM-CSF}$ or $^{OVA}DC_{TNF}$ cells were eosinophilic (18% and 13% eosinophils respectively, $p<0.05$) relative to the $^{OVA}DC_{IL-10}$ -treated animals (Figure 5.3.2). On the other hand, the saline-treated ALD mice which had not been exposed to antigen for ≈ 9 months (versus the $^{OVA}DC_{TNF}$ and $^{OVA}DC_{GM-CSF}$ groups which were given immunostimulatory OVA-loaded DCs ≈ 8 months earlier) displayed a decided airway eosinophilia. The mice receiving the tolerogenic $^{OVA}DC_{IL-10}$ contained 82% and 75% fewer eosinophils than $^{OVA}DC_{TNF}$ - ($p=0.02$) and $^{OVA}DC_{GM-CSF}$ - ($p=0.025$) treated mice, respectively. There was no statistically significant difference between the saline- and $^{OVA}DC_{IL-10}$ -treated animal's airway eosinophilia, despite the apparent 46% reduction in mean values (Figure 5.3.2).

5.3.3 Antibody responses

In this experiment the mice were treated as above, and their circulating OVA-specific antibodies were periodically assessed until 8 months post-treatment, when the animals were exposed one last time to OVA aerosols 48hr before sacrifice. The circulating OVA-specific IgE levels of the $^{OVA}DC_{IL-10}$ -treated asthmatic mice progressively diminished, such that by 16 weeks they had declined 81% relative to saline-treated ALD mice ($p\leq 0.001$) and 77% and 73% relative

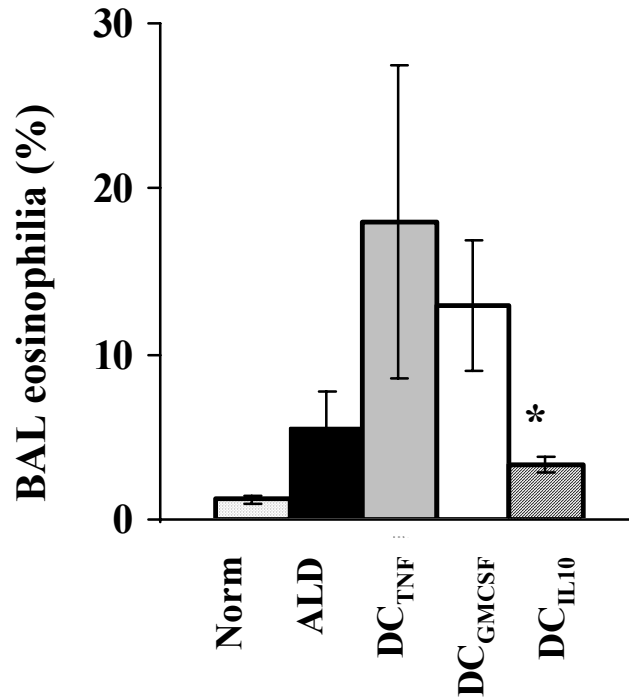


Figure 5.3.2 Long-term effect of a single $^{OVA}DC_{IL-10}$ -treatment on airway eosinophilia. BALB/c mice with severe ALD were treated with saline, $^{OVA}DC_{TNF}$, $^{OVA}DC_{GM-CSF}$ or $^{OVA}DC_{IL-10}$, but were harvested at 32 weeks, 48hr after a single aerosolized OVA challenge, and then assessed for airway cells as in Figure 5.2.2 (n=5). The controls were the saline-treated ALD mice and normal mice. * $p \leq 0.05$ versus the DC_{GM-CSF} and DC_{TNF} groups. All the DCs were cultured with OVA antigen before being delivered to animals. The above data is representative of three experiments performed.

to the $^{OVA}DC_{TNF-}$ and DC_{GM-CSF} -treated mice ($p \leq 0.001$; Figure 5.3.3, upper left). At the time of euthanasia (i.e., two days after their final allergen exposure) their plasma IgE levels remained substantially lower than those of the saline-treated ALD control (54% lower; $p \leq 0.001$), $^{OVA}DC_{TNF-}$ (22% lower; $p \leq 0.05$) or DC_{GM-CSF} -treated mice (60% lower; $p \leq 0.001$).

The OVA-specific IgG1 levels were significantly reduced in $^{OVA}DC_{IL-10}$ -treated relative to the saline-, $^{OVA}DC_{TNF-}$ and DC_{GM-CSF} -treated ALD mice ($p \leq 0.05$, Figure 5.3.3, upper right panel) both during the 32 week period of no antigen exposure as well after experiencing the allergen contact 48 hrs before sacrifice. Interestingly, we also found that the antibody levels started to rise between 19-29 weeks and on antigen challenge at 32 weeks an increase in antibody levels was found in all the groups.

Unlike earlier, where we found no difference in IgG2a antibody levels at 3 weeks, we observed noticeable decreases ($p \leq 0.001$, Figure 5.3.3, lower panel) in IgG2a levels at 8, 16 and 32 weeks in $^{OVA}DC_{IL-10}$ compared to saline-, $^{OVA}DC_{TNF-}$ and DC_{GM-CSF} -treated mice. Similarly, IgG2a Ab levels were augmented between 19-29 weeks and after exposure to the antigen the levels of antibody were increased in all the three groups but the relative decrease in $^{OVA}DC_{IL-10}$ compared to saline-, $^{OVA}DC_{TNF-}$ and DC_{GM-CSF} -treated groups remained statistically significant.

To summarize, the $^{OVA}DC_{IL-10}$ treatment significantly reduced the OVA-specific IgE over a period of time, although the OVA-specific IgG1 levels remain markedly low throughout the 8 months period, even though by 8 months they appeared to be increasing. In addition, IgG2a antibodies were considerably decreased in $^{OVA}DC_{IL-10}$ -treated compared to saline-, $^{OVA}DC_{TNF-}$ and DC_{GM-CSF} -treated animals although overall the levels were increased at 32 weeks. This data also suggests that like AHR, T cell tolerance also starts to wane sometime between 19-32 weeks.

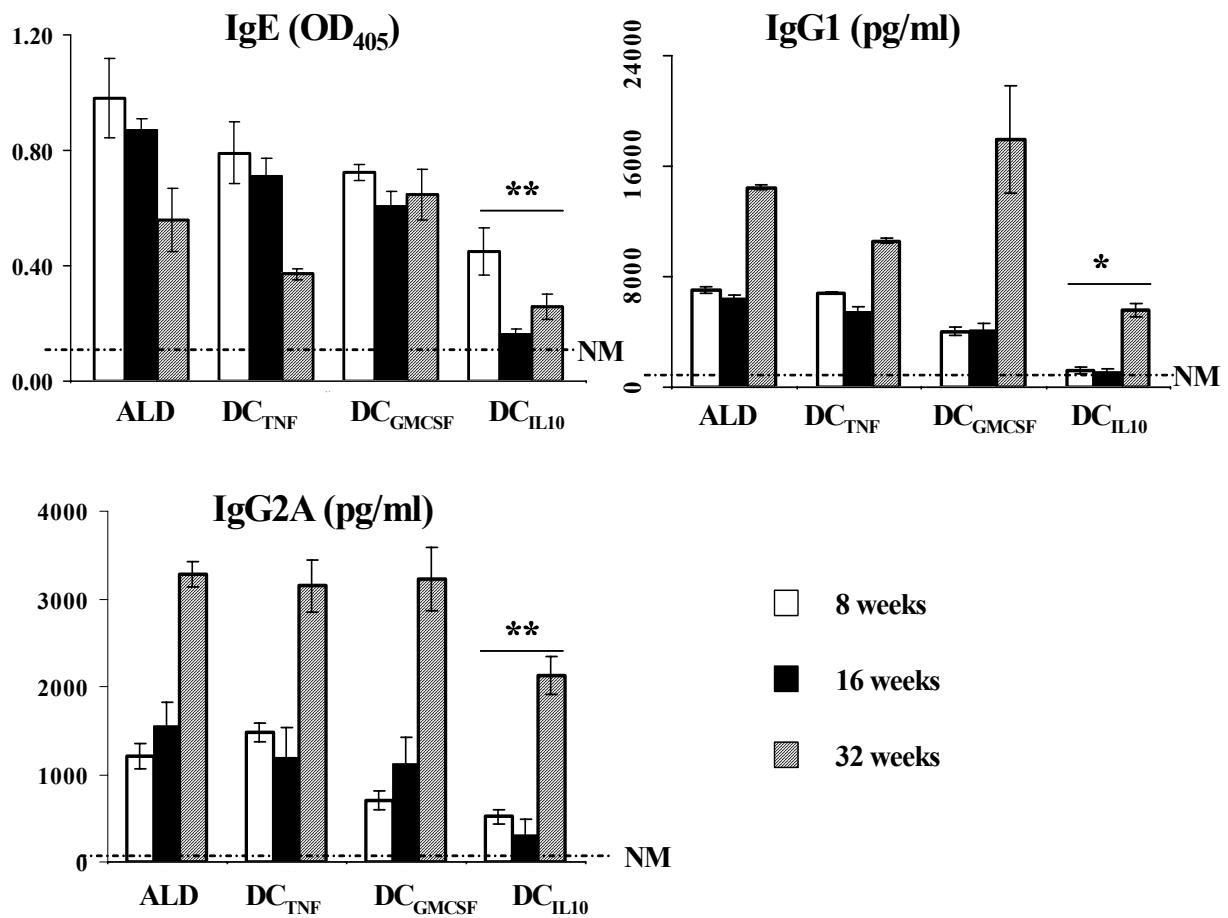


Figure 5.3.3 ^{OVA}DC_{IL-10} treatments significantly affect OVA-specific IgE and IgG1 as well as IgG2a antibody responses over a period of 8 months. BALB/c mice with severe ALD were treated as above and were harvested after 32 weeks and assessed for plasma or serum antibody levels as indicated in material and methods by OVA-specific ELISA. The controls were the saline-, ^{OVA}DC_{TNF}- and ^{OVA}DC_{GM-CSF}- treated ALD mice. IgE is expressed as OD₄₀₅ and IgG1 and IgG2a are expressed in pg/ml. * or ** = p ≤ 0.05 or p ≤ 0.01 versus each of the control groups. These are the results from one of three experiments (n=5/group)

5.3.4 Cytokine secretion in the bronchoalveolar lavage (BAL) fluid

The BAL levels of Th2 cytokines at this time (i.e. 8 months post treatment and 48hr after a single antigen exposure) were also markedly affected. The levels of IL-4, and IL-5 in the $^{OVA}DC_{IL-10}$ treatment group were dramatically reduced ($p \leq 0.001$) compared to the saline-, $^{OVA}DC_{TNF}$ and $^{OVA}DC_{GM-CSF}$ treatments (Figure 5.3.4). The BAL levels of IL-9 in $^{OVA}DC_{IL-10}$ -treated animals were considerably reduced compared to the saline- ($p=0.002$) and $^{OVA}DC_{GM-CSF}$ -treated groups ($p=0.025$). The levels of IL-13 ($p=0.03$) were also notably lower compared to saline-treated ALD controls, but not significantly lower than the $^{OVA}DC_{TNF}$ - and $^{OVA}DC_{GM-CSF}$ -treated ALD controls (Figure 5.3.4). These results indicate that $^{OVA}DC_{IL-10}$ treatment significantly decreased the Th2 cytokine response in the airways over a period of 8 months. Besides, this it also suggests that $^{OVA}DC_{GM-CSF}$ acts as an immunoreactive population that possibly matures in the inflammatory environment of lungs such as that of asthmatic lungs in our mouse model.

5.3.5 Lung parenchymal cytokine secretion

We also assessed the release of Th2 cytokines over 48 hrs by lung parenchymal mononuclear cells, as noted above. As seen earlier at 3-week time point, the saline-treated ALD mice showed no differences compared to the $^{OVA}DC_{IL-10}$ -treated mice (except IL-9). The IL-9 secretion by the cells from the $^{OVA}DC_{IL-10}$ -treated mice was considerably decreased ($p \leq 0.001$, Figure 5.3.5). While the mean values for IL-4 and IL-13 were $\approx 51\%$ ($p > 0.05$) and 99%

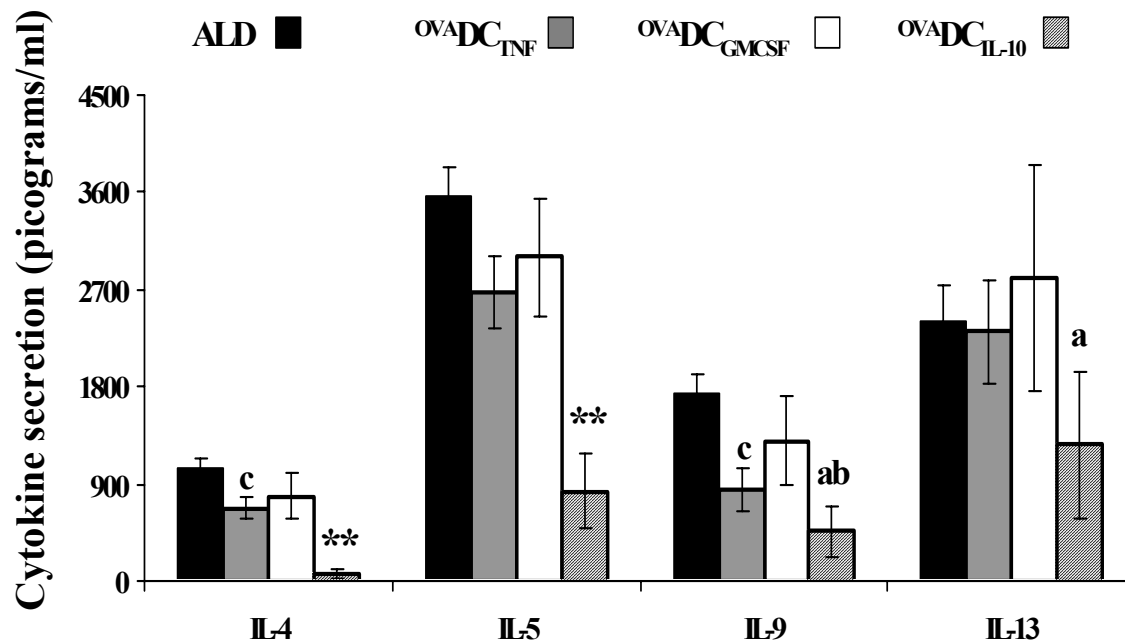


Figure 5.3.4 A single $\text{OVA DC}_{\text{IL-10}}$ treatment ameliorates airway Th2 cytokine responses to recall allergen challenge after a period of 8 months. BALB/c mice with severe ALD were treated as above and were harvested at 32 weeks, 48 hrs after a 1% OVA challenge and assessed for BAL fluid cytokine levels by ELISA, as noted in the materials and methods. The controls were the saline-, $\text{OVA DC}_{\text{TNF}}$ - and $\text{OVA DC}_{\text{GM-CSF}}$ -treated ALD mice. ** = $p \leq 0.001$ versus all the controls. “a” and “c” = $p \leq 0.05$ versus ALD group, “b” = $p \leq 0.05$ versus $\text{OVA DC}_{\text{GM-CSF}}$ treated animals. (n=5/group).

($p=0.055$) lower, respectively, compared to saline-treated ALD mice but neither was statistically significant. The levels of IL-5 were not significantly different between the saline- and $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated groups. As discussed earlier, we did not titrate the DC: T cells due to cell number constraint. However, the lung cells of the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated mice released much less IL-4, IL-5, IL-9 and IL-13 in culture than those from the immunoreactive $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ -treated animals (each, $p \leq 0.001$, Figure 5.3.5). Similarly the cells from the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated mice released less IL-4, IL-5, IL-9 and IL-13 in culture compared to those from the $\text{OVA}^{\text{DC}}_{\text{TNF}}$ -treated animals (each, $p \leq 0.05$). Thus, this data shows that the treatment of allergic mice with tolerogenic cells ameliorates lung parenchymal responses.

These data not only indicate that the tolerance was in place at this time but also that, despite an 8 month interval since their last allergen exposure, a fully functional population of T effector-memory (T_{EM}) cells presumptively resided in the lungs or associated lymphoid tissues of the saline-treated ALD animals. In addition, the tolerance which was not observed at 3 weeks in the lung parenchyma was now clearly obvious and differential effects of all the three DC populations were quite evident.

To summarize, the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treatments reduced airway eosinophilia, decreased the OVA-specific antibody responses and diminished the lung Th2 cytokine responses as determined at 8 months post-treatment, although the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatment had transient effects on the AHR.

5.4 Effect of various routes and repeated administration of $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ therapy in tolerance induction.

Our data indicated that a single $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatment can transiently ablate AHR, and has long-term effects on eosinophilic inflammation and Th2 responses, although the allergen-specific

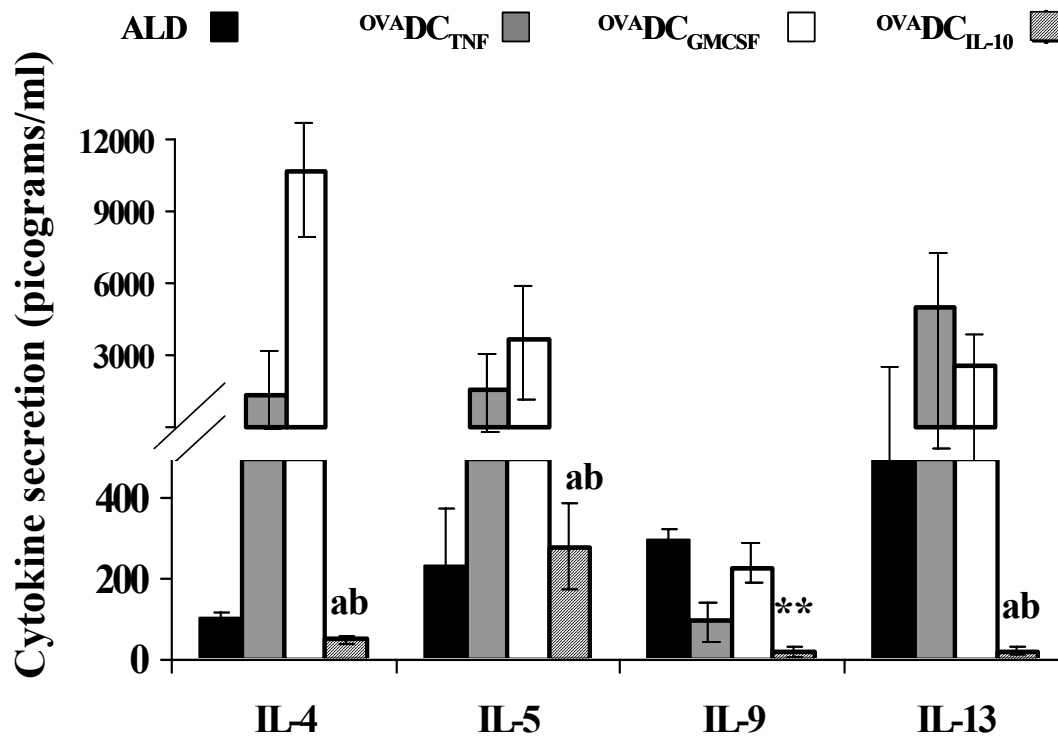


Figure 5.3.5 DC_{IL-10} treatments suppressed the lung parenchymal Th2 responses after an 8 month period. BALB/c mice with severe ALD were treated as above and were harvested at 32 weeks, 48 hrs after exposure to 1% OVA aerosols. Their lung parenchymal mononuclear cells were cultured for 48 hrs without exogenous OVA challenge, and the culture supernatant were assayed for Th2 cytokine levels by ELISA. *** = $p \leq 0.001$ relative to the saline treated ALD group. “a”= $p \leq 0.001$ versus OVA DC_{GM-CSF} group and “b”= $p \leq 0.05$ versus OVA DC_{TNF} -treated group. (n=5/group).

B cell responses were somewhat more resistant to tolerization, (e.g. 3-week data). Also the long-term experiment suggested that $^{OVA}DC_{IL-10}$ -induced tolerization was progressively effective. Therefore we wanted to know whether we could augment the tolerogenic effects of $^{OVA}DC_{IL-10}$ treatment by repeated delivery of these cells. Secondly, the transience of its effect on AHR led us to investigate whether this was a direct effect of $^{OVA}DC_{IL-10}$ delivery into the airways. We therefore also assessed the effect of delivering DC to distant sites, but in this experiment we also explored whether repeated treatments would be better than a single treatment.

We introduced 1×10^6 $^{OVA}DC_{IL-10}$ either intratracheally, intraperitoneally (i.p.) or subcutaneously (s.c.) a total of four times (weeks 0, 2, 4, and 6), then challenged the recipients with OVA aerosols at week 8, and sacrificed them the 2 weeks later. The controls were treated with saline or $^{OVA}DC_{TNF}$.

5.4.1 Airway Hyperresponsiveness (AHR)

We assessed the AHR of all animals at week 3 and again at 8 weeks, just prior to sacrifice. As expected, we found that repeated intratracheal $^{OVA}DC_{IL-10}$, but and not the $^{OVA}DC_{TNF}$ or saline, treatments normalized the bronchial responsiveness at 3 weeks and these remained normalized at 8 weeks. The AHR with i.p. treatments was also normalized by 3 weeks and remained normalized by week 8 with respect to the respective saline and $^{OVA}DC_{IL-10}$ treatments. The AHR of the ALD mice receiving DC_{IL-10} s.c. was completely unaffected, even after four treatments (Figure 5.4.1). This clearly indicates that repeated $^{OVA}DC_{IL-10}$ administration, whether by i.t. or i.p. route, reverses bronchial hyperreactivity. This suggests that

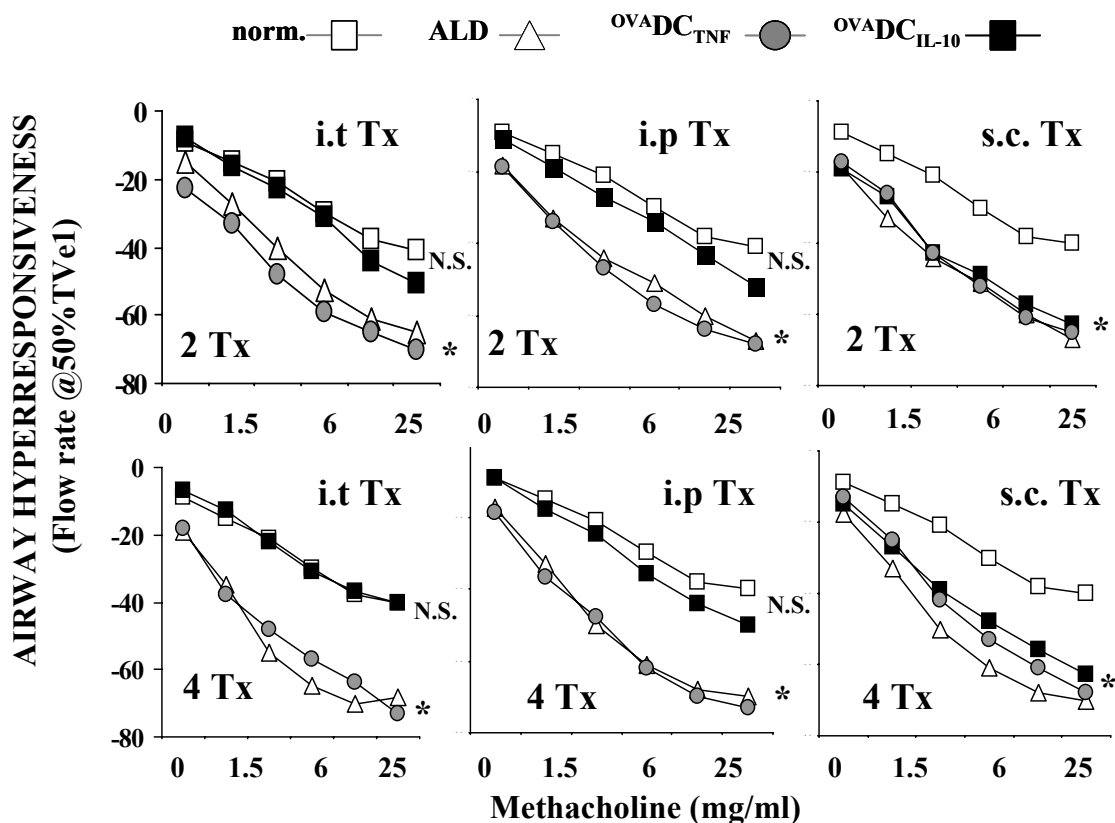


Figure 5.4.1 Repeated intratracheal or intraperitoneal but not subcutaneous administration of $^{OVA}DC_{IL-10}$, reverses AHR. ALD was induced as earlier indicated in the materials and methods. Tolerogenic DCs ($^{OVA}DC_{IL-10}$) or mature DCs ($^{OVA}DC_{TNF}$) were given either i.t., i.p. or s.c., every second week for a total of 4 treatments. Control mice received equal volumes of saline via the same route as their matched DC recipients. We measured the AHR a week after each treatment by head-out whole body plethysmograph as indicated earlier. These are the results of one experiment performed (n=5 mice/group). 1x and 4x represents AHR after first and fourth treatment. *, $p \leq 0.05$ for DC_{IL-10} versus saline-treated ALD mice. N.S. = non-significant, relative to normal mice. The data is presented as mean values. The SEM ranged between 0.5-5% for all graphs.

this effect either is not related to direct effects of IL-10 secretion by the tolerogenic DC within the airways, or perhaps that the DC given i.p. may somehow traffic by themselves into the lungs.

5.4.2 Airway eosinophilia

Repeated airway delivery of $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ reduced airway eosinophilia following allergen challenge by $\approx 90\%$ ($p=0.003$), versus the matched saline controls and by $\approx 91\%$ ($p=0.04$) compared to the matched $\text{OVA}^{\text{DC}}_{\text{TNF}}$ group (Figure 5.4.2). In addition, while the i.p. treatments reduced eosinophilic inflammation by 83% ($p=0.02$), versus saline treatments and 76% ($p=0.01$) compared to the matched $\text{OVA}^{\text{DC}}_{\text{TNF}}$ group, the s.c. treatments were less effective ($\approx 55\text{-}60\%$ reductions) versus the airway $\text{OVA}^{\text{DC}}_{\text{TNF}}$ ($p=0.05$) and saline ($p=0.052$) treatment groups. Additional significant difference ($p=0.01$) was observed between the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ i.t. and s.c. treatments. Thus repeated delivery of tolerogenic cells augmented the reductions in eosinophilia in the airways of severely allergic mice.

5.4.3 Antibody responses

The repeated intratracheal $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatments reduced the IgE and IgG1 responses by 90% and 93%, respectively, compared to the saline-treated ALD animals, and by 85% and 88%, respectively, versus the matched $\text{OVA}^{\text{DC}}_{\text{TNF}}$ treatments (Figure 5.4.3). This was a substantially better outcome than that following a single treatment (Figure 5.2.3). OVA-specific IgG2a responses were reduced by 91% and 85% compared to their respective saline and $\text{OVA}^{\text{DC}}_{\text{TNF}}$ groups. Intraperitoneal delivery of $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ similarly tolerized the allergen-driven antibody

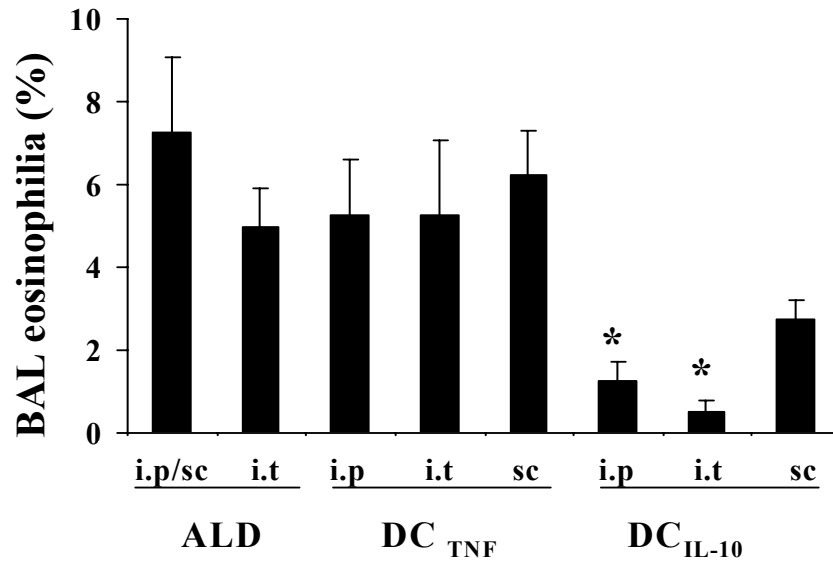


Figure 5.4.2 Repeated intratracheal or intraperitoneal but not subcutaneous administration of $^{OVA}DC_{IL-10}$, reverses airway eosinophilia. ALD mice which received tolerogenic DC ($^{OVA}DC_{IL-10}$), mature DC ($^{OVA}DC_{TNF}$) or saline treatments either i.t., i.p. or s.c., as described in Figure.5.4.1, were aerosolized with 1% OVA for 20 min at 8 weeks (2 weeks after the last DC delivery). After 48 hrs the mice were euthanized and BAL eosinophil responses were assessed. The controls were the saline- and $^{OVA}DC_{TNF}$ -treated ALD mice. There was no difference in controls given saline i.p. or s.c., so they are presented as one bar. * = $p \leq 0.05$ versus the saline- and $^{OVA}DC_{TNF}$ -treated group. These are the results of a single experiment performed (n=4 mice/group).

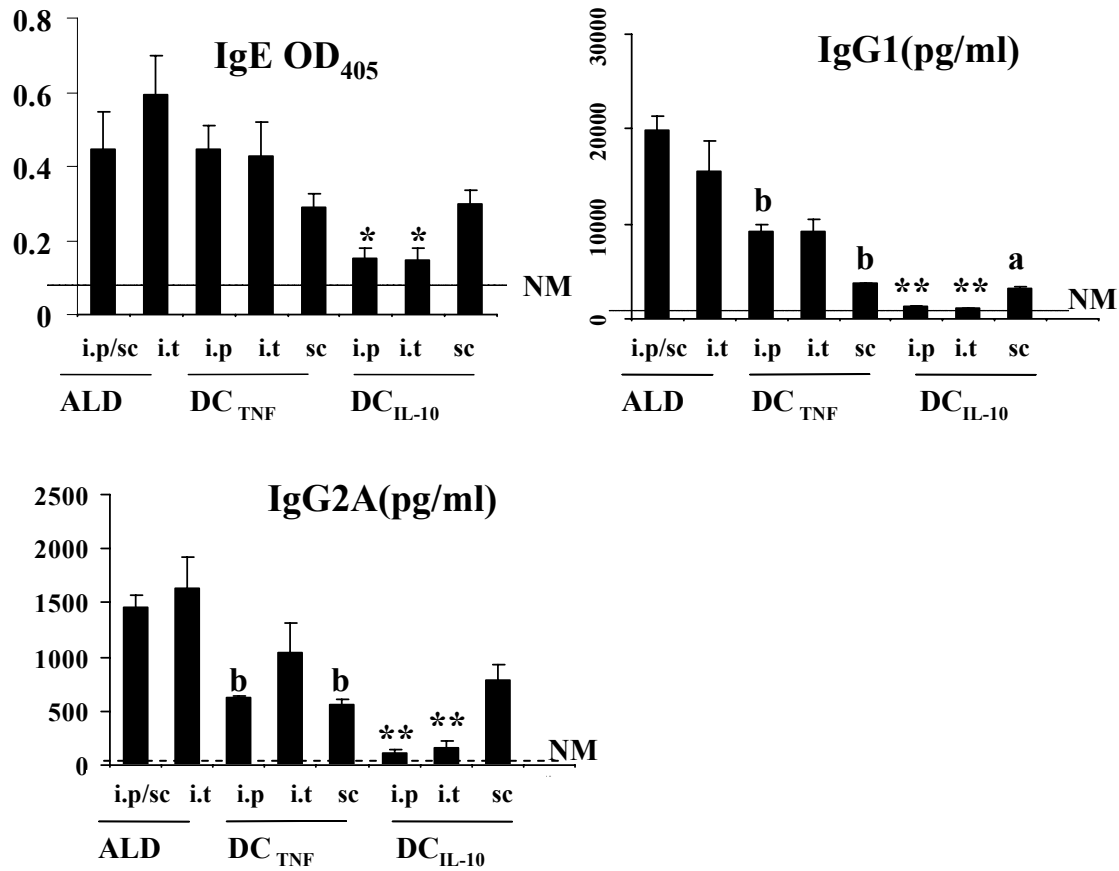


Figure 5.4.3 Repeated intratracheal or intraperitoneal but not subcutaneous administration of ^{OVA}DC_{IL-10}, ameliorates OVA-specific antibody responses. ALD mice which received tolerogenic DC (^{OVA}DC_{IL-10}), mature DC (^{OVA}DC_{TNF}) and saline treatments either intratracheally (i.t.), i.p. or s.c., as described in Figure.5.4.1, and were aerosolized and euthanized as indicated in Figure.5.4.2. OVA-specific ELISAs were done as explained in the materials and methods.* or ** = p≤0.05 or p≤0.01 compared to both saline and ^{OVA}DC_{TNF} treatments. “a” = p≤0.01 compared to respective the saline treatment and “b”= p≤0.01 compared to the respective saline treatment. OVA-specific IgE is expressed as OD₄₀₅ and IgG1 and IgG2a are expressed in pg/ml. These are results of one experiment performed (n=4/group).

responses, reducing IgE, IgG1 and IgG2a levels by 84%, 94%, and 87%, respectively, relative to the saline-treated animals, and by 84%, 86% and 82%, respectively, versus the $^{OVA}DC_{TNF}$ treatments. The s.c. delivery of $^{OVA}DC_{IL-10}$ was not nearly as effective, reducing the IgE, IgG1, and IgG2a responses by 41%, 84%, and 49%, respectively, versus the saline controls. Interestingly, the $^{OVA}DC_{TNF}$ groups given their cells via i.p. or s.c., not the i.t., route also showed significant decreases ($p \leq 0.01$) in IgG1 and IgG2a compared to their respective saline controls. In addition, significant differences were seen within the $^{OVA}DC_{IL-10}$ treatments given via different routes. $^{OVA}DC_{IL-10}$ given i.t. or i.p. showed significantly ($p \leq 0.05$) lower levels of IgE, IgG1 and IgG2a antibodies relative to the mice given the cells s.c. Thus, while the 8-week IgE and IgG1 responses had been relatively resistant to a single $^{OVA}DC_{IL-10}$ treatment (Figure 5.3.3), repeated treatments over 8 weeks brought these responses to near back-ground levels. In addition, the i.t. and i.p. routes tended to be better routes for effective therapeutic delivery of cells than was the s.c. route.

5.4.4 Cytokine secretion in bronchoalveolar lavage (BAL) fluid

Overall, the airway Th2 responses (i.e., BAL IL-4, -5, -9, and -13) to allergen challenge were reduced by +/-80% versus saline controls in the animals repeatedly given tolerogenic DC, irrespective of the route (Figure 5.4.4A). In addition, when comparing with matched $^{OVA}DC_{TNF}$ treatments, we found significant decreases in IL-4 and IL-5 when the DCs were given via airway and in IL-13 when the cells were given via i.p. delivery. No significant differences were seen between the $^{OVA}DC_{IL-10}$ and $^{OVA}DC_{TNF}$ treatments when the cells were given s.c.

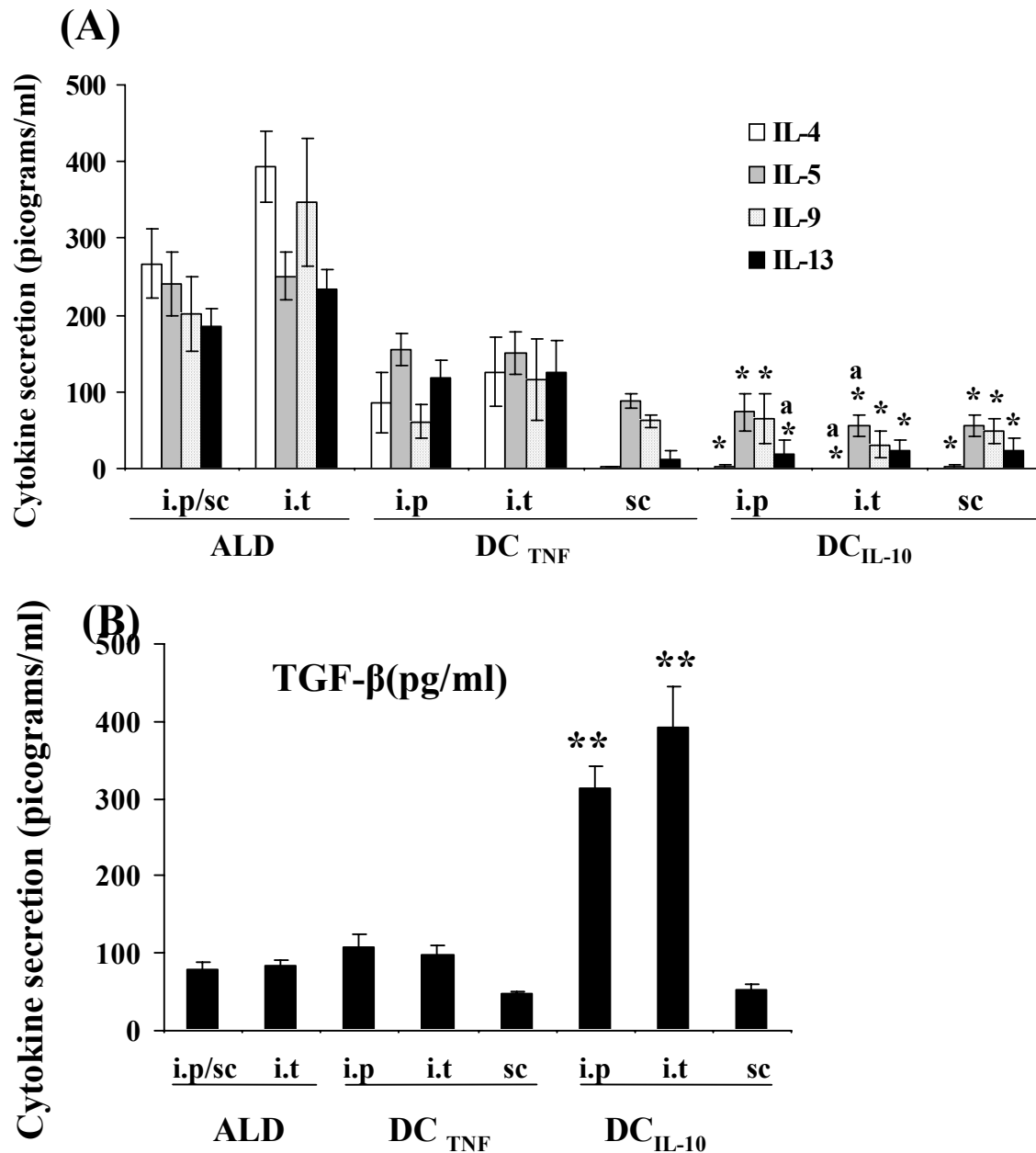


Figure 5.4.4 Repeated intratracheal or intraperitoneal but not subcutaneous administration of ^{OVA}DC_{IL-10}, reduces BAL Th2 responses. ALD mice which were treated as in Figure 5.4.1 were aerosolized and euthanized. The BAL fluid Th2 cytokine levels were assessed by ELISA as indicated in materials and methods. “*”= $p \leq 0.05$ compared to their respective saline treatments, “a”= $p \leq 0.05$ versus their respective ^{OVA}DC_{TNF} group. ** $p \leq 0.001$ versus saline-treated ALD mice. These are results of the one experiment performed (n=4 mice/group).

Intriguingly, the levels of TGF- β in the airways of the i.t. and i.p. DC-recipient mice were upregulated substantially (>4-fold), while this did not occur in the s.c. DC treatment (Figure 5.4.4B). Specifically, BAL TGF- β levels in the saline-, i.t. $^{OVA}DC_{TNF}$ - and i.t. $^{OVA}DC_{IL-10}$ -treated ALD mice were 84, 99 and 338 pg/ml, respectively. These findings suggested that repeated airway $^{OVA}DC_{IL-10}$ treatments substantially ameliorate Th2 responses and that TGF- β could potentially be involved.

5.4.5 Pulmonary parenchymal responses

We also assessed Th2 cytokine secretion by enzymatically dispersed lung parenchymal mononuclear cells, using ELISAs on 48 hr lung culture supernatants. The lung cells of the i.t. $^{OVA}DC_{IL-10}$ -treated mice released much less IL-4, IL-5, IL-9 ($p \leq 0.05$) compared to the saline-treated animals. In addition, $^{OVA}DC_{IL-10}$ -treated mice given cells i.t. also showed significantly lower levels for all cytokines relative to their matched $^{OVA}DC_{TNF}$ treatments (Figure 5.4.5). On the other hand, comparing with matched $^{OVA}DC_{TNF}$ treatments, we found significant decreases in IL-4 and IL-13 ($p=0.04$ each, i.p.-treated mice) and in IL-5 and IL-13 (s.c. delivery), while no differences were detected between the saline- and the matched $^{OVA}DC_{IL-10}$ -treated mice. Although the saline-treated groups had had no antigen exposure for ≈ 10 weeks prior to the final preharvest aerosolization, their parenchymal responses were significantly exaggerated compared to mice given $^{OVA}DC_{IL-10}$ intratracheally. Therefore, repeated delivery of $^{OVA}DC_{IL-10}$ markedly reduced the lung responses, and the intratracheal delivery route led to overwhelming reductions in lung parenchymal mononuclear cell responses compared to the i.p. and s.c. routes of delivery.

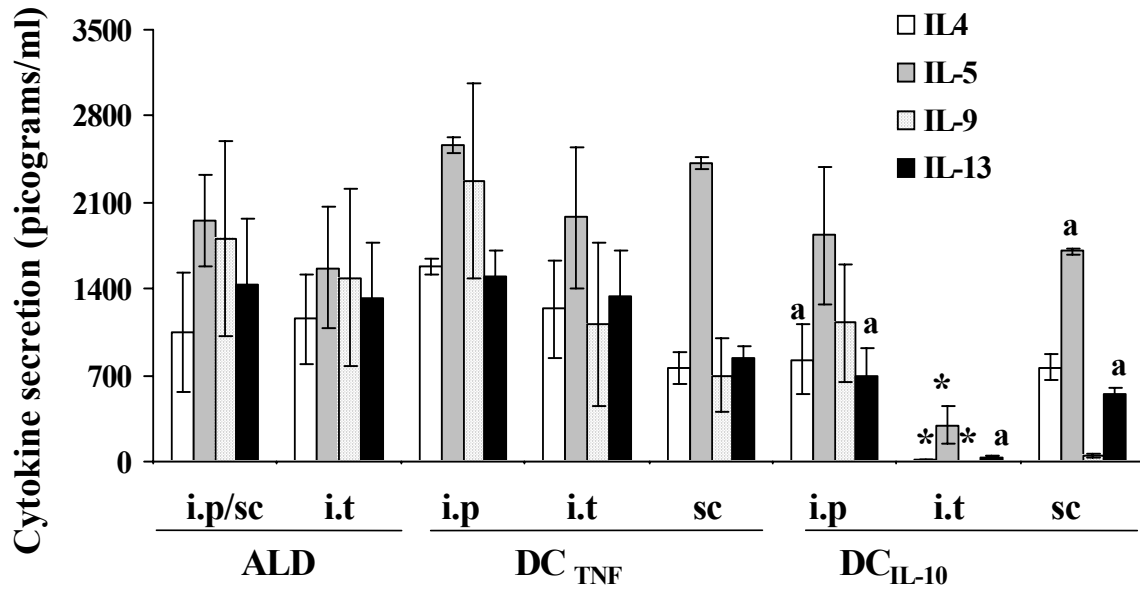


Figure 5.4.5 Repeated intratracheal or intraperitoneal but not subcutaneous administration of $^{OVA}DC_{IL-10}$, reduces lung parenchymal Th2 responses. ALD mice which received tolerogenic DC ($^{OVA}DC_{IL-10}$), mature DC ($^{OVA}DC_{TNF}$) or saline treatments either intratracheally (i.t.), i.p. or s.c., as described in Figure 5.4.1 were aerosolized and euthanized as indicated in Figure 5.4.2. The lung mononuclear cells of the OVA challenged mice were assessed for cytokine secretion as in Figure 5.2.5. “*”= $p \leq 0.05$ compared to their respective saline and $^{OVA}DC_{TNF}$ treatments and “a”= $p \leq 0.05$ versus their respective $^{OVA}DC_{TNF}$ group. These are results of one experiment performed (n=4 mice/group).

5.4 Mechanisms mediating DC_{IL-10} induced tolerance *in vitro* and *in vivo*

To dissect out the mechanisms involved in DC_{IL-10} induced tolerance we initially wanted to establish a relevant *in vitro* model. Allergic lung disease was established in mice as before, but then they were rested for 3 weeks to allow the allergen-activated T cells in the lungs to differentiate into a central memory T cell state (T_{CM}). At that point CD4-positive cells were MACS-purified from the lungs, spleens and lymph nodes and cocultured for 72 hrs with OVA⁺DC_{IL-10}, OVA⁺DC_{TNF} or OVA⁺DC_{GM-CSF}. In our initial experiments, we used OVA⁺DC_{IL-10} and OVA⁺DC_{TNF} but we found that the mice treated with the latter cells displayed some tolerance as determined by BAL Th2 cytokine levels. While OVA⁺DC_{GM-CSF} behaved as immunostimulatory cells in the *in vivo* experiments, we also assessed their effects on *in vitro* memory T cell responses, as compared to the putatively tolerogenic OVA⁺DC_{IL-10}. To determine the roles of putative effector mechanisms in this system we added recombinant IL-2, anti-IL-10R or -TGF- β antibodies or the IDO antagonist 1-methyltryptophan (1-MT) to the cultures. The culture supernatants were analyzed for our target cytokines.

5.5.1 Effectiveness of OVA⁺DC_{IL-10} treatment *in vitro*

As expected, a general trend towards reductions in Th2 cytokines was observed in OVA⁺DC_{IL-10} treated cultures. The levels of IL-5 and IL-13 were significantly lower ($p \leq 0.05$) in OVA⁺DC_{IL-10}-supplemented cultures compared to the OVA⁺DC_{TNF} cultures. Also, the addition of OVA⁺DC_{IL-10} to the T_{CM} cells markedly reduced their expression of IL-4, -5 and -13, relative to parallel OVA⁺DC_{GM-CSF} co-cultures (Figure 5.5.1.1). There was $\approx 57\%$ reduction in IL-9 release in

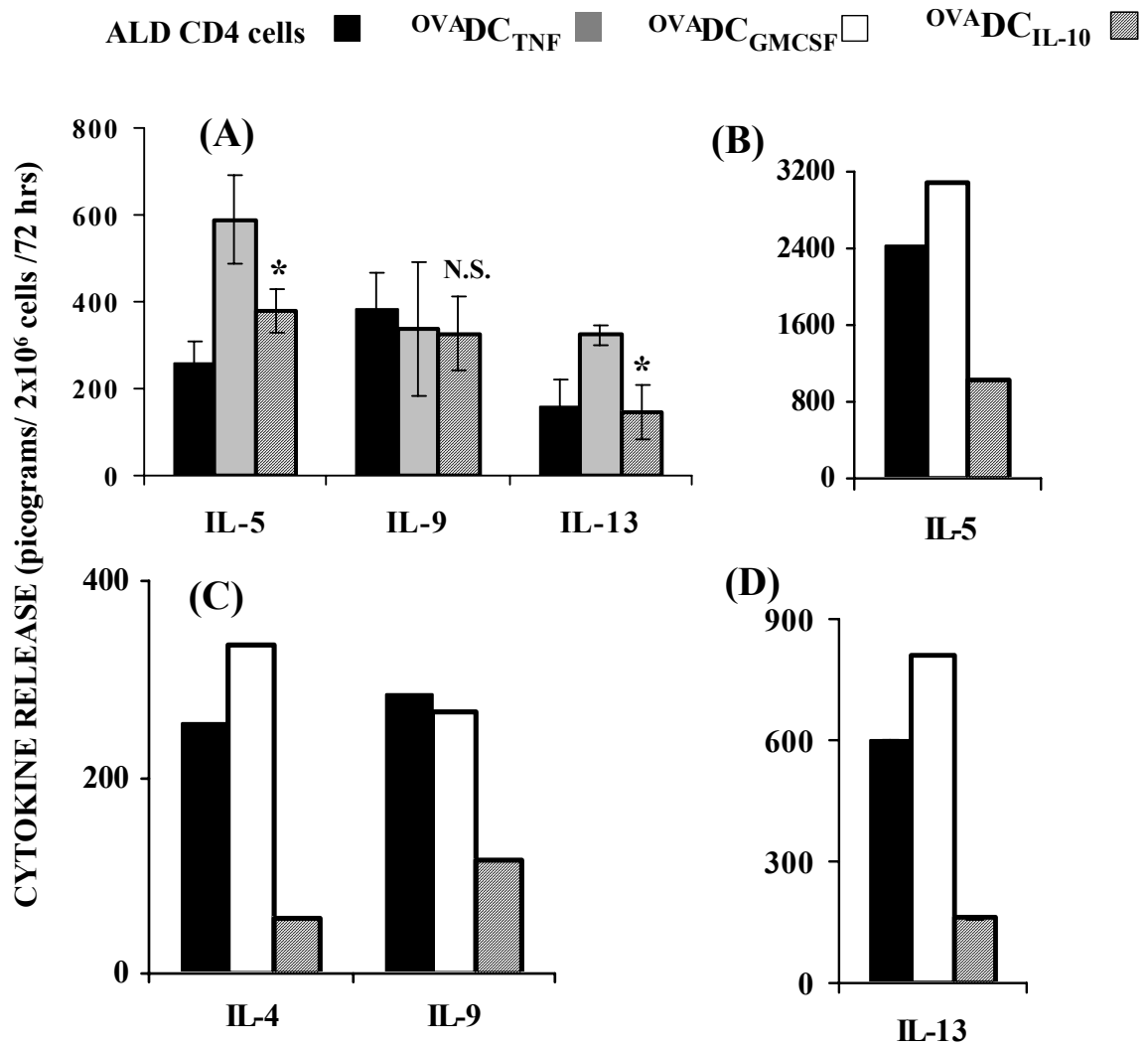


Figure 5.5.1.1 DC_{IL-10} do not activate memory T cells *in vitro*. ALD was induced in the mice as indicated in the materials and methods (section 4.3). After a period of 3 weeks, MACS-purified $CD4^+$ T_{CM} cells were harvested from the lungs, spleens and lymph nodes of these ALD mice and cocultured for 72 hrs with $OVA DC_{TNF}$, $OVA DC_{GM-CSF}$ or $OVA DC_{IL-10}$. The purity of the $CD4^+$ cells was 90-94%. The first graph (A) represents the mean of 3 experiments. The other graphs (B, C and D) are from a single experiment (stats was not done because samples were pooled). The culture supernatants were analyzed for the indicated cytokines by ELISA. * $p \leq 0.05$ compared to $OVA DC_{TNF}$ -matched controls

the $^{OVA}DC_{IL-10}$ co-cultures compared to the $^{OVA}DC_{GM-CSF}$ cultures, although we did not see this in $^{OVA}DC_{TNF}$ cultures.

The addition of rIL-2 did in part reverse the DC_{IL-10} -dependent T cell unresponsiveness (Figure 5.5.1.2). The mean values for expression were increased by $\approx 45\%$ with respect to IL-5 and by $\approx 38\%$ for IL-9, while that for IL-4 secretion was increased by 68% in the IL-2-supplemented cultures, but none of these were statistically significant. However, the IL-13 response was significantly increased ($p \leq 0.05$). Supplementation of cultures with anti-IL-10R or -TGF- β antibodies had no discernible effect on this tolerance (Figure 5.5.1.3). Unlike what we observed previously with $CD8\alpha^+$ splenic $DC-T_{CM}$ co-cultures (Gordon *et al*, 2005), addition of the competitive IDO inhibitor 1-MT to the $DC_{IL-10}-T_{CM}$ cell cultures also had no significant effects on their release of these mediators (Figure 5.5.1.3). Unlike $^{OVA}DC_{TNF}$ or $^{OVA}DC_{GM-CSF}$, $^{OVA}DC_{IL-10}$ did not support activation of T_{CM} from ALD mice. Whether this was because they induced or initiated a bona fide state of immunologic tolerance cannot be deduced with assurance based on this data, but the observation that IL-2 in part reversed this effect is consistent an anergic process being present, as shown earlier (Essery, Feldmann *et al*. 1988). Although, rIL-2 supplementation caused partial reversal of unresponsiveness anti-IL-10R or -TGF- β antibodies or 1-MT addition had no evident effect in this system.

5.5.2 *In vivo* assessment of DC_{IL-10} induced tolerance

We next turned our attention to the effector mechanisms of tolerance in our asthmatic animals. We treated them with some of the above antagonists starting day 12 after the $^{OVA}DC_{IL-10}$ transfers, the time when evidence of tolerance first became physiologically discernible (AHR first became affected), and continued through until they were sacrificed 48 hrs after airway

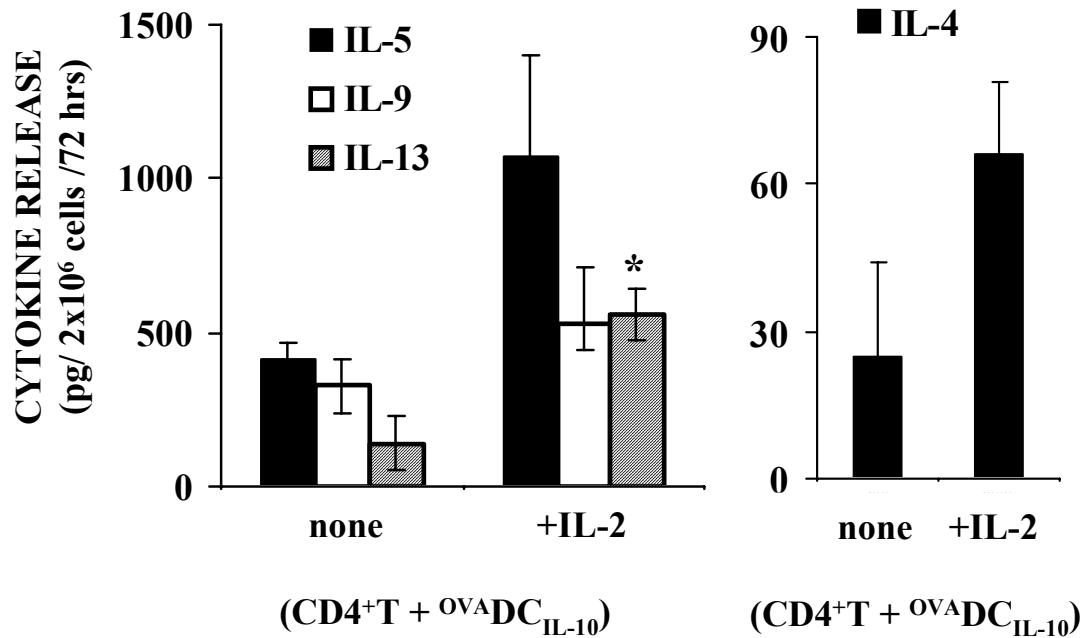


Figure 5.5.1.2 Addition of IL-2 partially reverses Th2 responses *in vitro*. ALD was induced in mice as indicated in the materials and methods (section 4.3). After a period of 3 weeks MACS-purified CD4⁺ T_{CM} cells were harvested from the lungs, spleens and lymph nodes of these mice and cocultured with OVA DC_{IL-10} with or without supplementation with recombinant IL-2 (10 U/ml). The 72 hr culture supernatants were analyzed for the indicated cytokines by ELISA. This data represents mean of 3 experiments. *= p≤0.05 compared to OVA DC_{IL-10} controls.

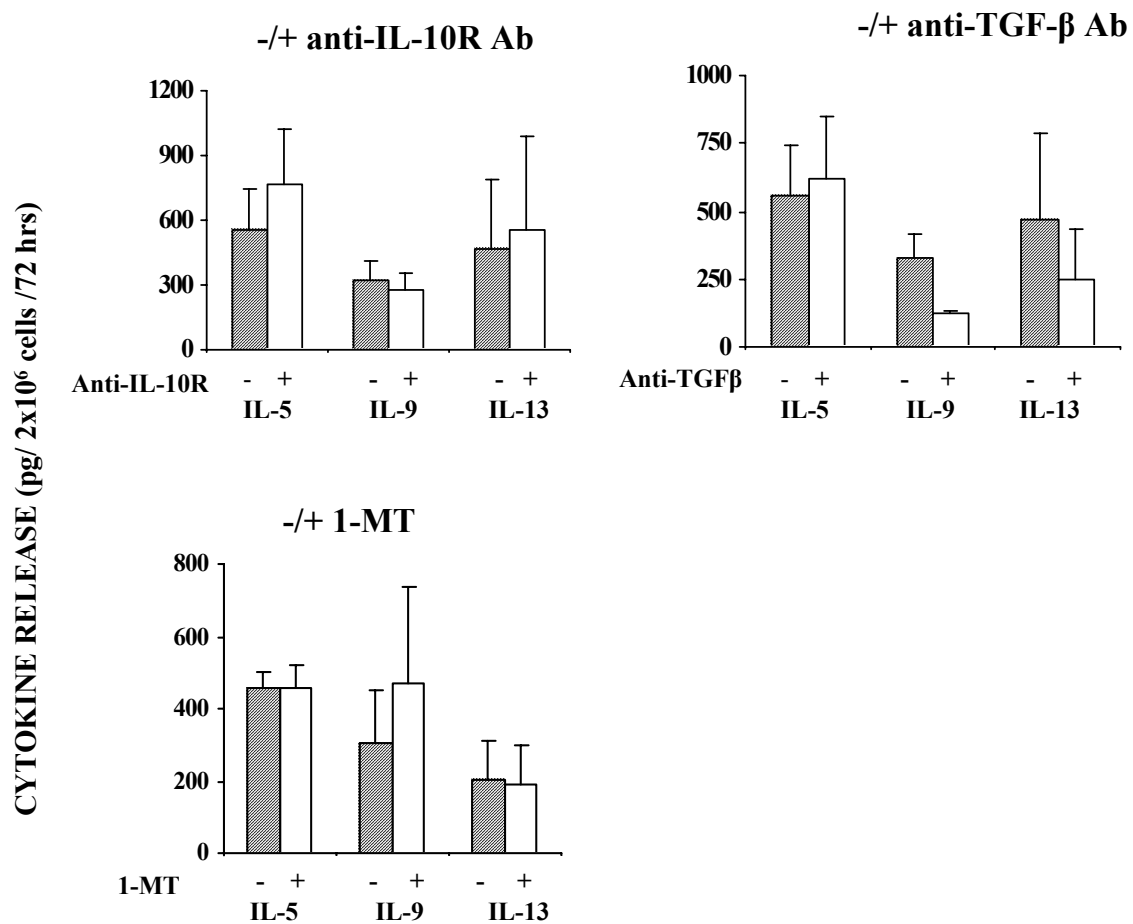


Figure 5.5.1.3 ^{OVA}DC_{IL-10}-induced T cell inactivation is not mediated by IL-10, TGF-β or IDO *in vitro*. ALD was induced in the mice as indicated in material and methods (section 4.3). After a period of 3 weeks MACS-purified CD4⁺ T_{CM} cells were harvested from the lungs, spleens and lymph nodes of these mice and cocultured with ^{OVA}DC_{IL-10} with or without supplementation with anti-IL-10 Ab (25 μg/ml), anti-TGF-β Ab (25 μg/ml) and 1-methyltryptophan (1-MT; 200 μM). The 72 hr culture supernatants were analyzed for the indicated cytokines by ELISA. This data represents mean of 3 experiments.

allergen challenge (day 21). Anti-IL-10R (5 mg/kg B-wt), -TGF- β (4 mg/kg B-wt), or isotype control antibodies were injected i.p., while continual delivery 1-MT (reported release rate, 10 mg/day) or placebo pellets were surgically implanted subcutaneously. These levels of these antagonists were chosen based on their efficacy in other model systems (Sakurai, Zou *et al.* 2002; Liu, Hu *et al.* 2003; Neptune, Frischmeyer *et al.* 2003; Shi, Pan *et al.* 2003). The mice were harvested two days after allergen aerosol challenge and were analyzed as earlier for airway function, pulmonary and systemic responses.

5.5.2.1 Neutralization with Anti-IL-10R and -TGF- β antibodies (Abs)

There were no significant effects of either anti-IL-10R or -TGF- β Ab treatments delivered from day 12-21 post-^{OVA}DC_{IL-10} treatment on the animal's AHR, compared to their respective isotype controls (Figure 5.5.2.1A). There were no differences between normal, ^{OVA}DC_{IL-10} and anti-IL-10R Ab-treated mice ($p \geq 0.05$) and each was markedly different (each, $p \leq 0.01$) from the saline-treated ALD controls. While, the anti-TGF- β treated mice were different ($p \leq 0.05$) from the normal mice and DC_{IL-10}-treated mice ($p \leq 0.05$), they were not different from the saline-treated ALD controls or TGF- β isotype control mice.

The airway eosinophilia was $\approx 95\%$ increased by the anti-IL-10R antibody treatments, relative to the isotype control ($p = 0.02$; Figure 5.5.2.1B, left panel) while it was unaffected by the anti-TGF- β treatments. There were no discernible effects of either anti-IL-10R or -TGF- β treatments regarding OVA-specific IgE levels compared to their respective isotype controls

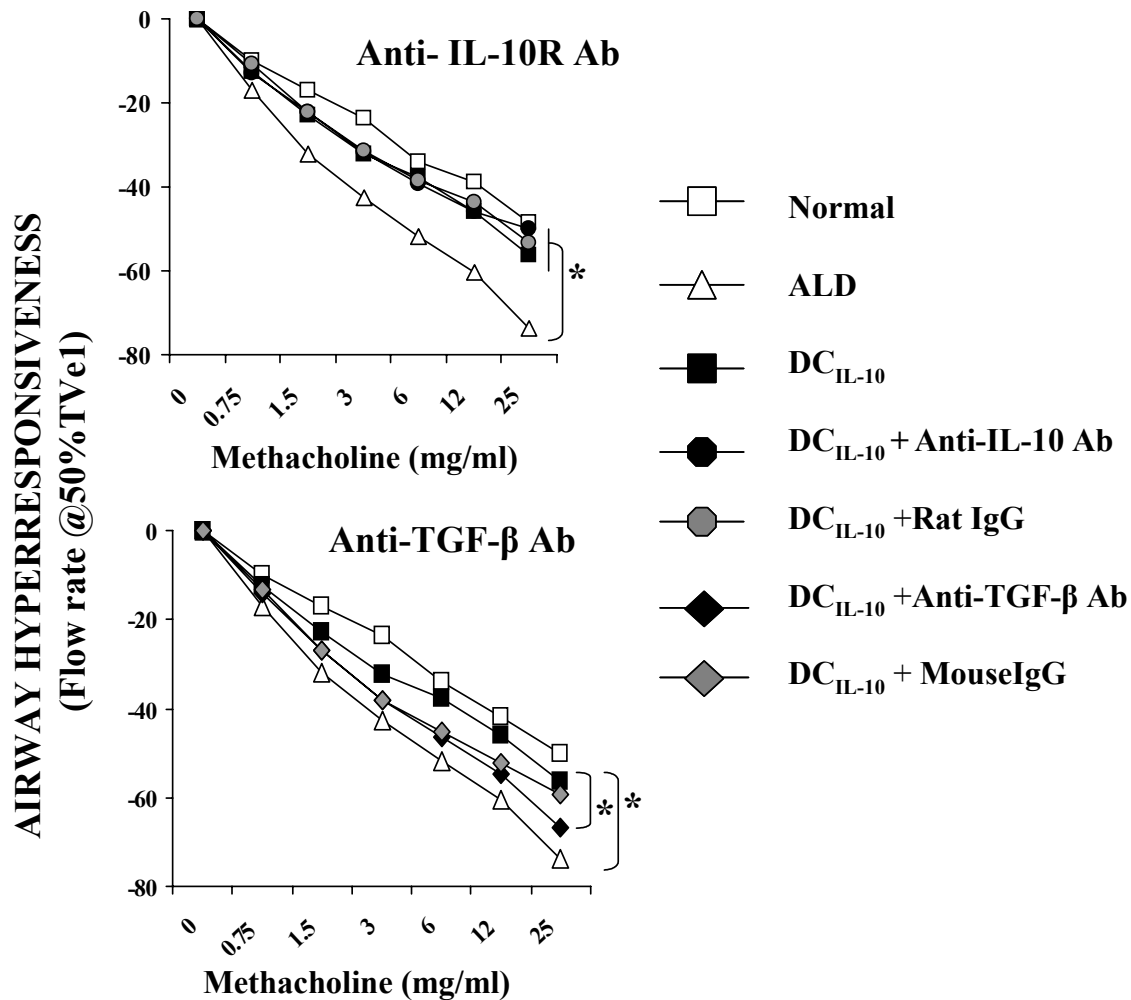


Figure 5.5.2.1A AHR of ALD mice is not affected by antagonism of IL-10 or TGF- β from day 12-21 post-^{OVA}DC_{IL-10} treatment. Anti-IL-10R (5 mg/kg B-wt, upper panel), -TGF- β (4 mg/kg B-wt, lower panel), or isotype control antibodies were injected i.p. from day 12-21 after ^{OVA}DC_{IL-10} treatment and AHR was measured at 3 weeks as in Figure 5.2.1. The data are expressed as the airflow rate at the 50% point in the expiratory cycle. The control groups are normal mice and saline-treated ALD mice. This experiment was done one time and each group contained 5-6 animals. * $p \leq 0.05$. The data represents mean values and SEM ranges between 1-3%.

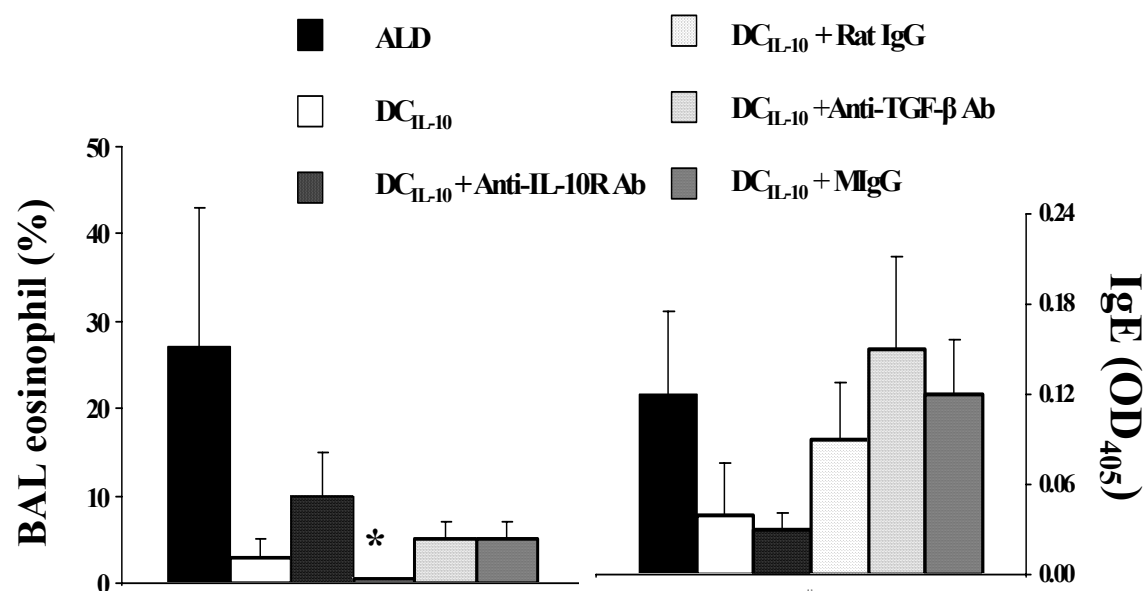


Figure 5.5.2.1B Neutralizing anti-IL-10R, but not anti-TGF-β antibodies, affect airway eosinophilia but not OVA-specific IgE. As noted in Figure 5.5.2.1A anti-IL-10R and anti-TGF-β-treated ALD mice were sacrificed 48 hr after aerosol exposure and assessed for eosinophils in the bronchoalveolar lavage fluids (left panel). To determine the percentage of each cell type, 200 cells were counted on each of the 4 slides per group (n=5-6). The OVA-specific IgE antibody levels (right panel) were assessed by ELISA as described in the materials and methods. The controls included were the saline-treated ALD mice (positive control) and DC_{IL-10}-treated isotype control antibody-injected mice. * = p ≤ 0.05 versus the anti-IL-10 Ab group. This experiment was done one time only. (n=5-6/group).

(Figure 5.5.2.1B, right panel). While the anti-IL-10R, but not anti-TGF- β , antibody treatments altered the BAL fluid mean values for IL-4 (by 95%) and IL-9 (by 64%; $p=0.056$) relative to the isotype control Abs, these were not statistically significant. The increases in BAL IL-5 levels (51%; $p=0.02$) and IL-13 (60%; $p=0.016$) were significant (Figure 5.5.2.1C, A). The BAL fluid cytokine secretion was indistinguishable between the anti-TGF- β and its isotype control mice (Figure 5.5.2.1C, B). We also assessed the release of Th2 cytokines by parenchymal cells from the lungs of our treated animals. We found that mean values for IL-4, IL-5, IL-9 and IL-13 were not elevated by either anti-IL-10R or anti-TGF- β Ab treatments, versus their matched isotype controls (Figure 5.5.2.1D).

To summarize, the neutralization treatments with anti-IL-10R antibody reversed the airway eosinophilia and BAL Th2 responses partially while anti-TGF- β did not affect the tolerance, suggesting that IL-10 secretion might be involved in $^{OVA}DC_{IL-10}$ -induced tolerance.

5.4.2.2 Role of indoleamine-2, 3-dioxygenase (IDO) in DC_{IL-10} -induced tolerance.

The impact on tolerance of inhibiting IDO with the competitive antagonist 1-MT was assessed using slow release polymer pellets which were surgically implanted s.c. IDO inhibition did not affect AHR (Figure 5.5.2.2A, left panel) of the $^{OVA}DC_{IL-10}$ -treated ALD mice (mean values from 4 experiments are presented in the graph as we did not have enough cells to run the $^{OVA}DC_{IL-10}$ controls).

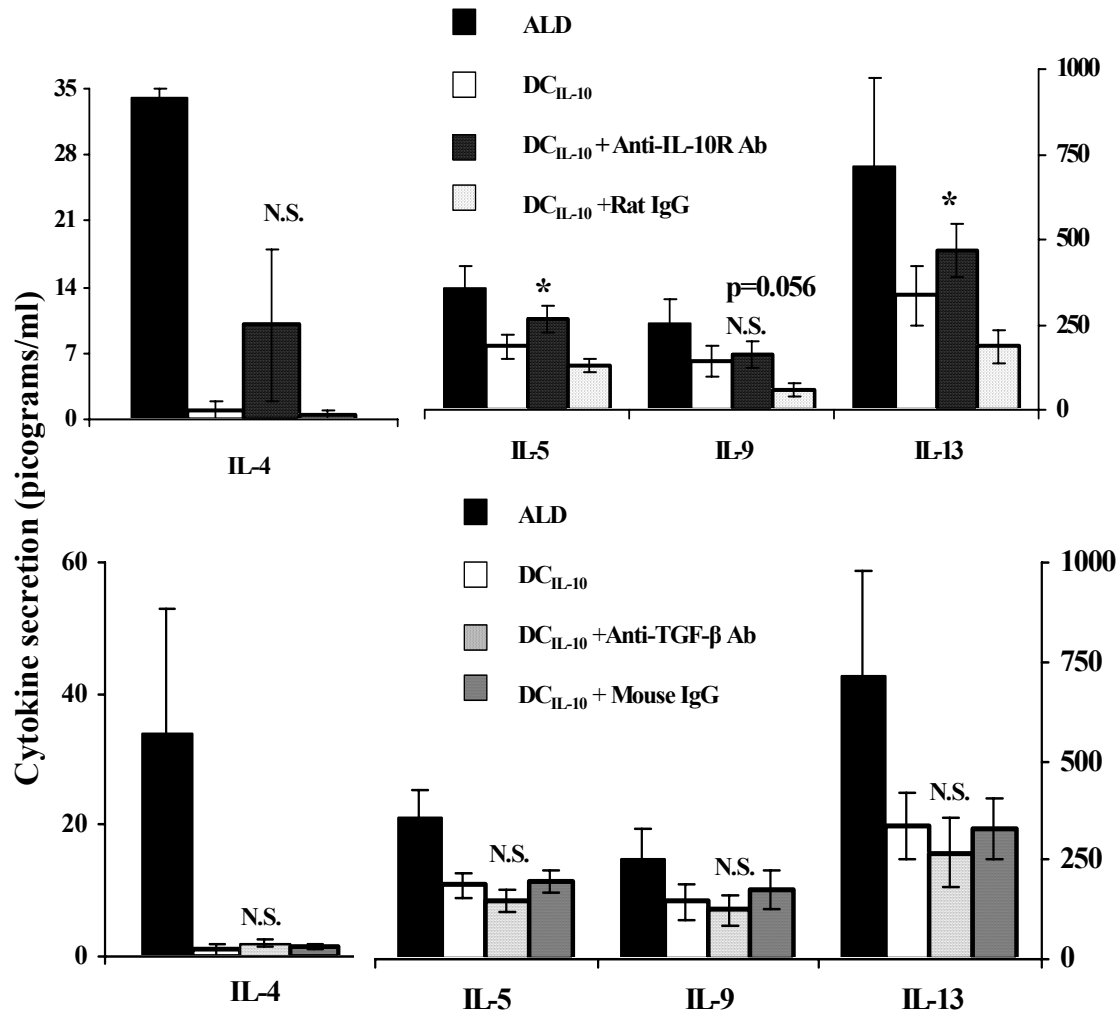


Figure 5.5.2.1C Neutralizing level of anti-IL-10R, but not anti-TGF- β antibodies partially affects BAL Th2 cytokine secretion. The ALD mice (n=5-6/group) as treated earlier with anti-IL-10R Ab or anti-TGF- β Ab were sacrificed after aerosol exposure and assessed for BAL fluid Th2 cytokine levels by ELISA as in the materials and methods. The specific antibody-treated groups were compared with their respective isotype controls. * = $p \leq 0.05$ and N.S. = non-significant relative to the matched isotype controls.

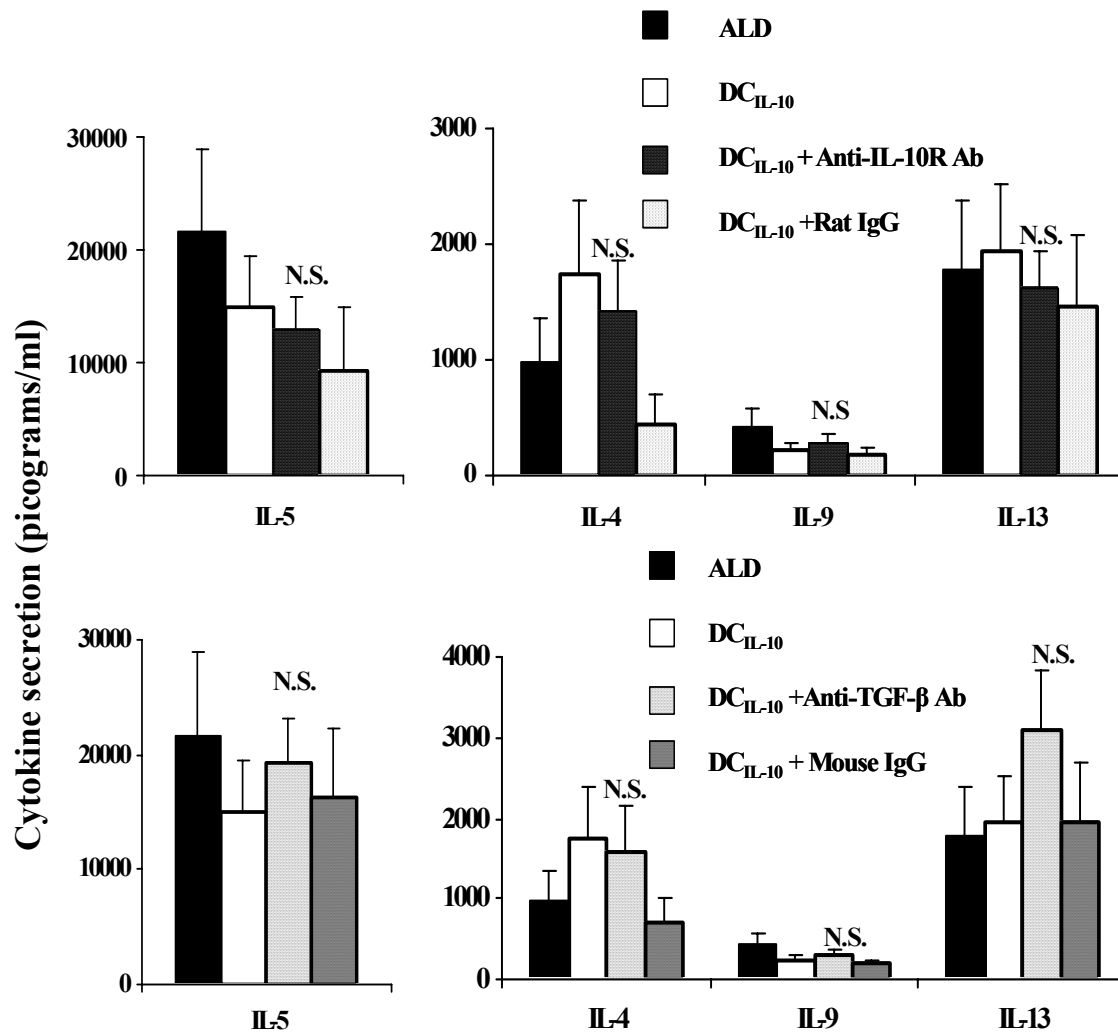


Figure 5.5.2.1D Neutralizing levels of anti-IL-10R or anti TGF- β antibodies, do not affect lung parenchymal Th2 cytokine levels. The ALD mice (n=5-6/group) as treated earlier with anti-IL-10 Ab or anti-TGF- β Ab were sacrificed after aerosol exposure and assessed for lung Th2 cytokine levels by ELISA as in the materials and methods. The specific antibody-treated groups were compared with their respective isotype controls. N.S. = non-significant relative to matched antibody-treated group.

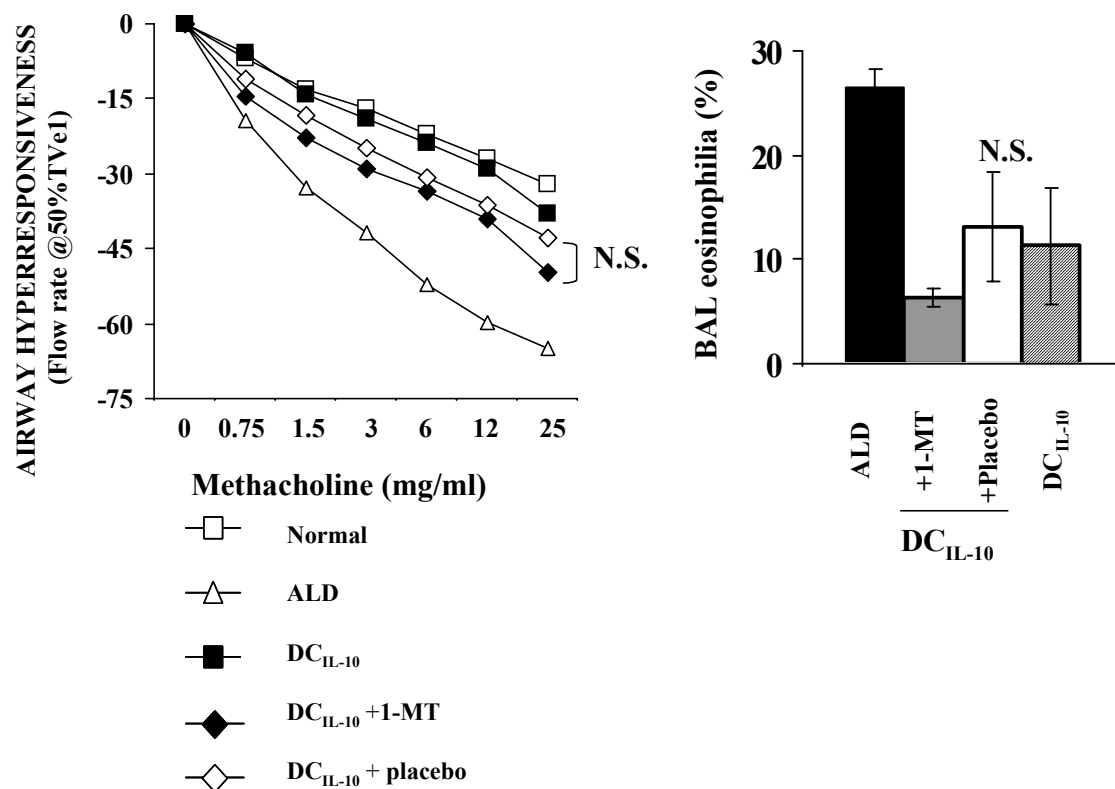


Figure 5.5.2A 1-methyl-DL-tryptophan (1-MT) treatments affected neither AHR nor airway eosinophilia. DC_{IL-10}-treated ALD animals (n=5) were surgically implanted with slow release polymer pellets impregnated with 1-MT (release rate 10 mg/mouse/day) or placebo pellets s.c. AHR was estimated weekly for 3 weeks and airway eosinophils were assessed in BAL fluids. This experiment was done once. DC_{IL-10} values are the mean values from 4 other experiments as we didn't have DC_{IL-10} controls. N.S. = non-significant relative to 1-MT treated mice. The AHR data is represented as mean values; and SEM ranged between 1-3%.

Similarly, $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -induced tolerization of airway eosinophilia was not reversed in the 1-MT-treated animals compared to placebo-treated animals (Figure 5.5.2.2A, right panel). IgE levels were not significantly ($p=0.07$) affected by 1-MT treatment. The IgG1 levels were lower in the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ and placebo groups ($p<0.01$) compared relative to the 1-MT-treated mice. Nevertheless, considerable differences remained ($p=0.01$) in IgG1 levels between the saline-treated ALD and 1-MT groups (Figure 5.5.2.2B, lower left panel). OVA-specific IgG2a levels were also increased ($p<0.05$) in the 1-MT-treated groups versus the placebo controls (Figure 5.5.2.2B, lower right panel), but these were not different from those of the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated animals. Inhibition of IDO was also associated with considerable increases in BAL fluid levels of IL-5 ($p<0.001$), IL-9 ($p=0.02$) and IL-13 ($p<0.001$) (Figure 5.4.2.2C) compared to the placebo-treated animals. The $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated mice were considerably different from the 1-MT-treated mice with regards to BAL IL-9 and IL-13 levels only. However, the 1-MT treatments did not fully suppress tolerance as the 1-MT-treated mice still expressed lower levels of IL-9 ($p=0.04$) and IL-13 ($p=0.03$) relative to the saline-treated ALD controls. IL-4 values were below the detectable limits of the ELISA in this experiments so are not presented here.

We also assessed the expression of IDO within the lung tissues of our animals by qRT-PCR. At 3 wk after transfer of 1×10^6 $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ into asthmatic mice, the overall lung levels of IDO mRNA levels were expressed at levels two-fold greater than saline-treated ALD controls (Figure 5.5.2.2D). We confirmed that the PCR product was bonafide IDO by sequencing the gel purified product (Figure 5.5.2.2E). The sequenced product had 100% identity with the Gene Bank sequence for mouse IDO (NCBI transcript accession No. NM008324). Taken together, this

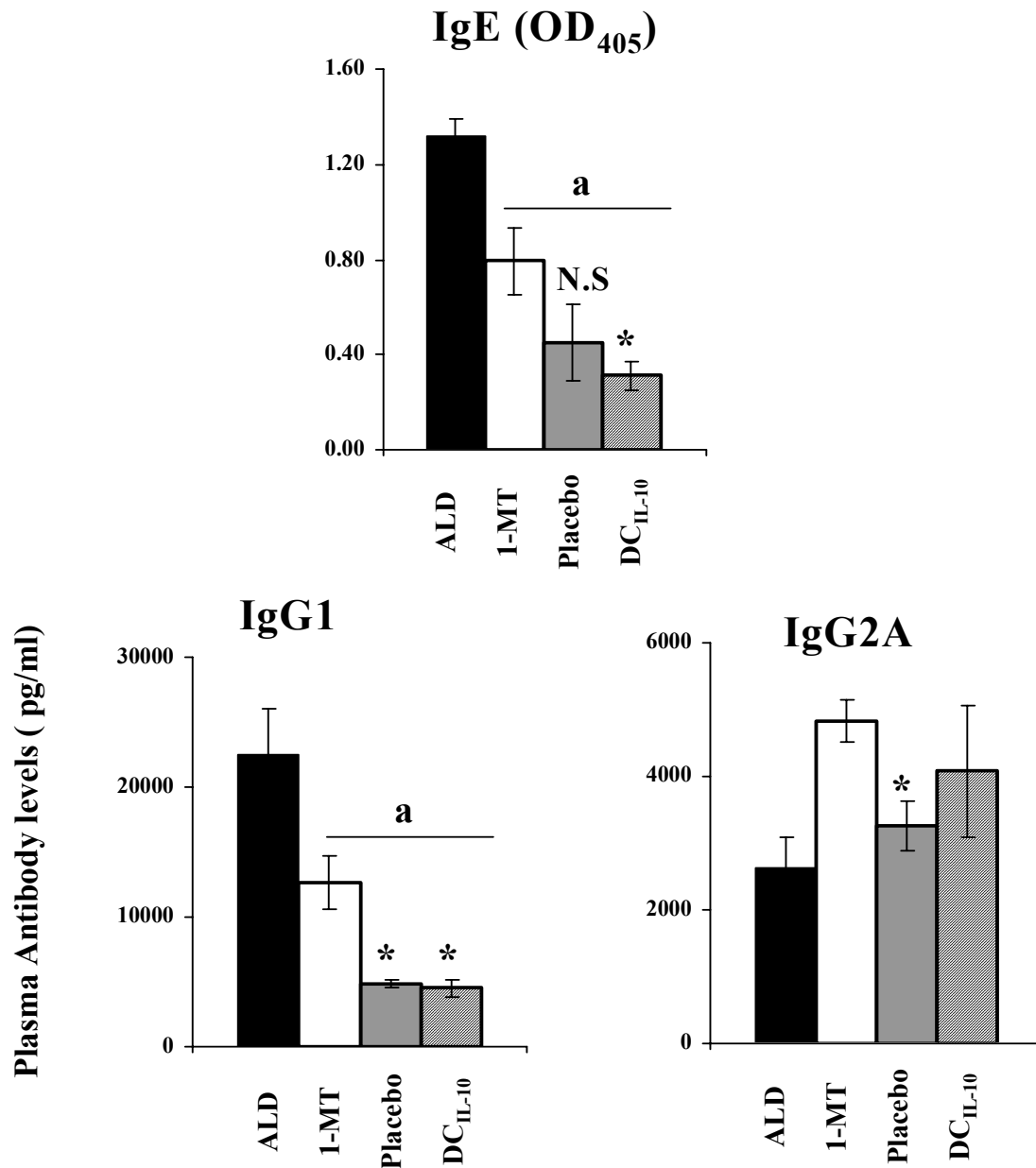


Figure 5.5.2.2B Effect of 1-methyl-DL-tryptophan (1-MT) on plasma antibody levels. ALD mice treated as in Figure 5.5.2.2A were assessed for OVA-specific plasma IgE, IgG1 and IgG2a levels by ELISA. IgE was expressed as OD₄₀₅ and IgG1 and IgG2a as pg/ml. * = $p \leq 0.05$ versus 1-MT group. “a” = $p \leq 0.05$ versus saline-treated ALD mice. N.S. = non-significant relative to 1-MT-treated animals.

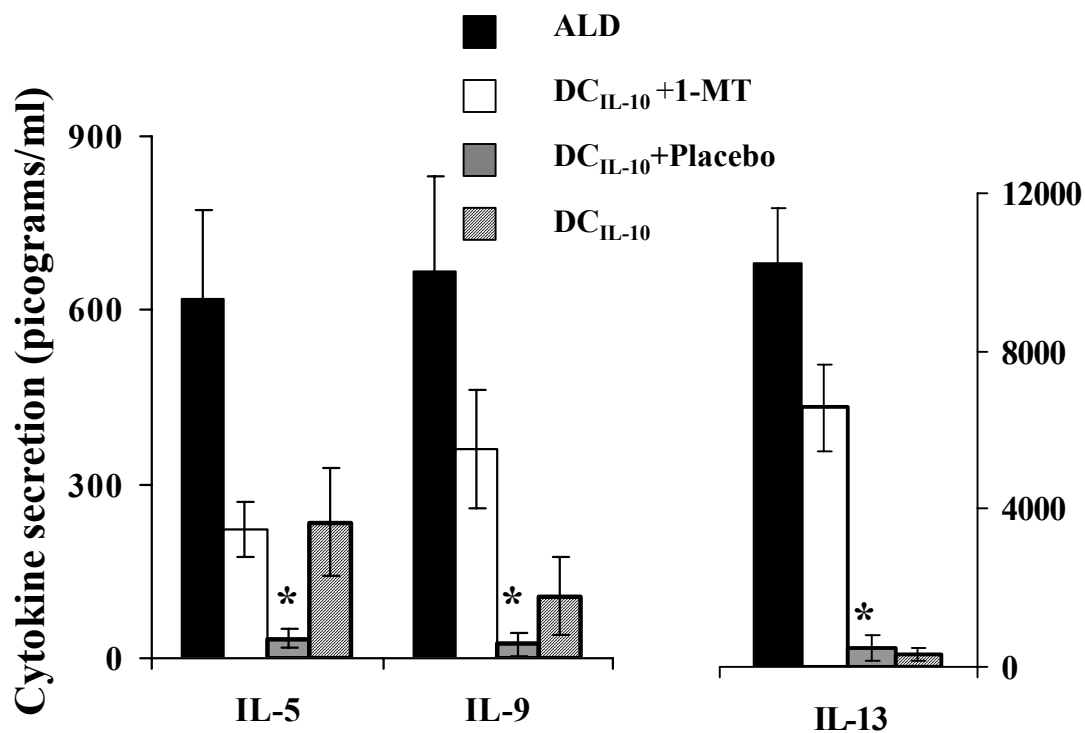


Figure 5.5.2.2C Indoleamine-2, 3-dioxygenase (IDO) plays a role in DC_{IL-10}-induced tolerance of asthma-like disease. DC_{IL-10}-treated ALD mice (n=5) were given s.c. slow release polymer pellets impregnated with 1-MT (release rate 10 mg/mouse/day) or placebo. The mice were aerosolized and sacrificed after 3 weeks and BAL fluids were assessed for the indicated Th2 cytokines by ELISA. * = $p \leq 0.05$ versus 1-MT controls. This experiment was done once. DC_{IL-10} values are the mean values from 4 other experiments as we didn't have DC_{IL-10} controls.

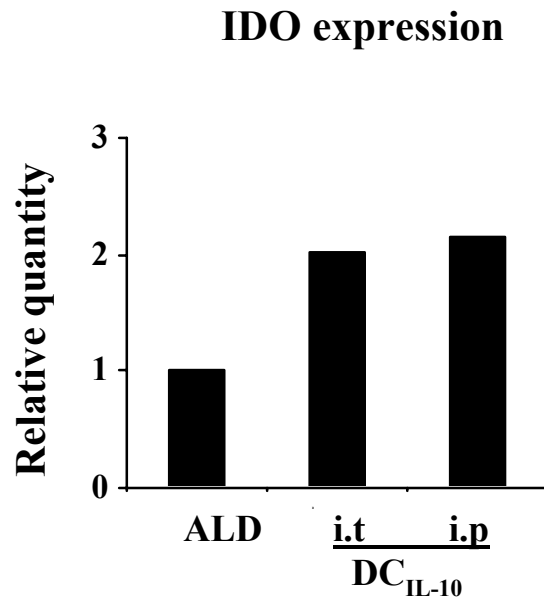


Figure 5.5.2.2D ^{OVA}DC_{IL-10}-treated ALD mice express increased levels of indoleamine-2, 3-dioxygenase mRNA. The ^{OVA}DC_{IL-10} treated ALD mice either i.t. or i.p. and saline controls were sacrificed after 3 weeks and the RNA purified from the lungs was analyzed for IDO expression by real time RT-PCR. The relative quantity of RNA is shown here.

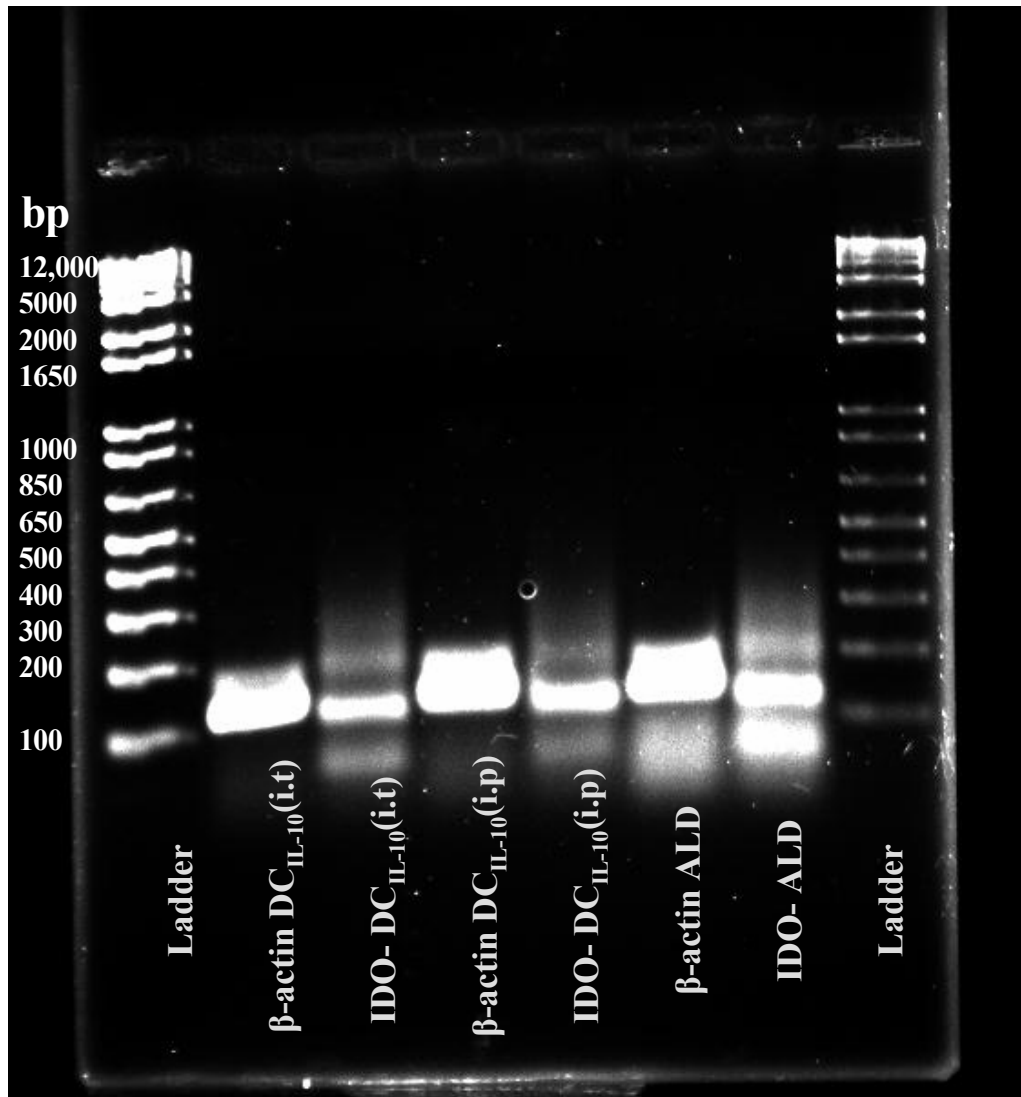


Figure 5.5.2.2E ^{OVA}DC_{IL-10} express indoleamine-2, 3-dioxygenase. The ^{OVA}DC_{IL-10} treated ALD mice either i.t. or i.p. and saline controls were sacrificed after 3 weeks and the RNA purified from the lungs was analyzed for IDO expression by real time RT-PCR as in Figure.5.5.2.2.E and we confirmed it by running the PCR product (120 base pairs (bp)) from the same experiment (Figure 5.5.2.2 D) on a 2% agar gel.

data suggested that IDO might play a role in the effector mechanisms of tolerance in $^{OVA}DC_{IL-10}$ -treated asthmatic animals.

In summary, we can conclude that $^{OVA}DC_{IL-10}$ -treatments led to reduction in *in vitro* expression of Th2 cytokines compared to the $^{OVA}DC_{GM-CSF}$ and $^{OVA}DC_{TNF}$ treatments and supplementation with rIL-2, but not anti-IL-10R or -TGF- β Abs or 1-MT, causes partial reversal of this effect. *In vivo*, anti-IL-10R antibody treatments partially reversed eosinophilia and BAL IL-5 and IL-13 levels, while inhibition of IDO partially reversed tolerization of the IgE, IgG1 and IgG2a and BAL Th2 cytokines.

6.0 DISCUSSION AND CONCLUSIONS

Allergic asthma is a global health problem, characterized clinically by varying degrees of intermittent and reversible airway obstruction, exaggerated airway responsiveness to a wide variety of stimuli and eosinophilic airway inflammation (Busse and Lemanske 2001). However, it is well recognized that these manifestations are driven by underlying Th2-type immune responses (Gavett, Chen *et al.* 1994). Conventional asthma treatments do not address the immunologic basis of this disease, but rather are primarily symptomatic in nature, targeting instead the AHR (e.g., bronchodilators) or pulmonary inflammation (e.g., steroidal and non-steroidal anti-inflammatory medications) (Lin and Casale 2002). As a less conventional approach, co-administration of allergen with CpG-oligodeoxynucleotides has been reported to reduce airway eosinophilia, Th2 cytokine induction, IgE production, and bronchial hyperreactivity in mouse models of asthma (Lin and Casale 2002), but does so by deviating of the allergic inflammatory responses to a Th1-dominant mode. This in itself carries significant health risks (i.e. allergen-driven hypersensitivity pneumonitis) (Kline, Waldschmidt *et al.* 1998). So far, specific allergen immunotherapy (SIT) is the only treatment available for specific allergy and is not very effective in patients with multiple sensitivities, which is the usual case (Adkinson, Eggleston *et al.* 1997). However, SIT is a long term treatment and has risks of side effects like urticaria, bronchospasms or anaphylaxis in the worst case scenario (1997). Taking all the facts into account, tolerization to allergen seems to be a more appropriate means of reducing asthma lung pathology.

DCs are the most potent stimulators of primary immune response known so far (Parra, Wingren *et al.* 1997) and as such they have been recognized as potentially important tools for immunotherapy and vaccine strategies (Porgador, Snyder *et al.* 1996; Cella, Sallusto *et al.* 1997). However, there is also increasing evidence that DC can perform a critical role in the induction and maintenance of peripheral tolerance (Mahnke, Schmitt *et al.* 2002) and this depends upon the micromilieu during the maturation of the DC. Under certain experimental conditions, the process of DC maturation can be altered. The immunosuppressive cytokine IL-10 has been implicated in the production of tolerogenic/regulatory DCs (Mahnke, Schmitt *et al.* 2002). The effects of IL-10 application have been investigated in various autoimmune and inflammation models, including experimental autoimmune encephalomyelitis (EAE), arthritis, pancreatitis, diabetes mellitus and experimental endotoxemia (Asadullah, Sterry *et al.* 2003). The anti-inflammatory effects of IL-10 have also been used to prolong graft survival in transplantation studies (Zuo, Wang *et al.* 2001). In humans it has been reported that IL-10 treatment of DCs enable them to inhibit both the allergen-specific Th1 and Th2 responses (Steinbrink, Wolfl *et al.* 1997; Bellinghausen, Brand *et al.* 2001). Also, in an OVA specific-T cell receptor transgenic model, IL-10-treated bone marrow-derived DCs (BMDC) have been shown to inhibit delayed-type hypersensitivity reactions in the footpads of animals vaccinated prophylactically or therapeutically (Muller, Muller *et al.* 2002).

Following this line of reasoning, we decided to characterize and examine the effect of antigen-loaded IL-10-treated BMDCs in severe allergic lung disease in a mouse model. We had previously shown that CD8 α ⁺ DCs can be used to \approx 50% reverse the asthma phenotype in mice (Gordon, Li *et al.* 2005). Others have shown that BMDC that are differentiated in TGF- β -containing medium are also modestly effective, blunting the localization of systemic allergen

responsiveness to the lungs following airway allergen challenge (Zhang-Hoover, Finn *et al.* 2005). So far, few groups have investigated the prophylactic role of IL-10- modulated DCs on Th2 responses in an allergic asthma model (Bellinghausen, Sudowe *et al.* 2006; Koya, Matsuda *et al.* 2007) and complete elicitation of IL-10- modulated DC therapy in established allergic lung disease still remains to be investigated.

Herein we have characterized IL-10-modulated, or tolerogenic, DCs (DC_{IL-10}) as compared to control populations of “immature” DC_{GM-CSF}. The mature populations of DC_{TNF} were also characterized for control purposes. It was found that DC_{IL-10} possessed a phenotype more like DC_{GM-CSF} on comparing the three populations. As had been reported earlier (Jonuleit, Schmitt *et al.* 2001) our FACS data revealed reduced levels of costimulatory markers CD40, CD54 and MHC-II in the DC_{IL-10} population. Studies done to date on DC differentiated with GM-CSF indicate the cells with purities of 60% by day 7-8 and 80-90% by day 10-12 were achieved, while 50-70% of cells differentiated with TNF- α or LPS have a mature DC phenotype (Lutz, Kukutsch *et al.* 1999). Masurier, *et al* reported the existence of two subpopulations of DCs cultured in GM-CSF, including MHC II^{hi} (30-50%) cells and MHC II^{low} (50-70%) cells. Of the MHC- II^{low} cells, 30% express CD11c, and 50% express 33D1 and both sub-populations are DEC205⁻ and F4/80⁺ (Masurier, Pioche-Durieu *et al.* 1999). We analyzed our DC populations as a whole for the presence of relevant markers and assessed alternate cell contamination. We designated our DC_{GM-CSF} as immature based on their avid phagocytic activity and lower levels of costimulatory markers. On the other hand, DC_{TNF} cells were grouped as mature because of their increased costimulatory marker expression, as observed earlier (Lutz, Kukutsch *et al.* 1999). Clearly there is no single specific marker for defining cultured BMDCs (e.g. they express macrophage markers Mac-1 and F4/80 and low levels of DC-specific markers), but the markers

we did use (CD40, CD54, CD80, CD86 and MHC-II) collectively define DC populations. The cultures were also tested for the presence of contaminating cells and were assessed for markers like CD19 (B cells; <8%) and CD14 (monocytes; <20%). We acknowledge that there is a possibility of other cells being present in the cultures but at the same time DCs can express CD19 (Bjorck and Kincade 1998; Baban, Hansen *et al.* 2005) as well as CD14 (Mahnke, Becher *et al.* 1997). Neutrophils comprised $3\% \pm 1$ of our cells. However, their ability to influence the outcomes in our experiments is likely very modest, in as much as even if they were introduced into asthmatic airways they would likely undergo apoptosis rapidly (e.g., within a day).

Like immature DC, our tolerogenic DCs possessed strong chemotactic responses to the inducible CC chemokine CCL3 (a CCR5 ligand), a key mediator of inflammatory and immune responses. This could have been instrumental in their homing to the airways of OVA-asthmatic animals (Appendix-1; data generated by Dr. H. Huang) when given by the i.p. route and directly to the inflammatory foci in the lungs when given via an i.t. route. DC_{IL-10} and DC_{GM-CSF} responded more weakly to the constitutively lymph node-expressed CC chemokine CCL19 (a CCR7 ligand, which is responsible for homing of maturing DCs to the lymphoid organs). Nevertheless, this indicates that a subpopulation of these cells would potentially have some capacity to migrate to, and prime T cells in, the lymphoid organs. It has been previously shown that these cells can induce tolerance on entry into the lymph nodes (Lin, Suri *et al.* 1998). Since the work reported in this thesis was completed, our lab has used ^{OVA}DC_{IL-10} labeled with the lipid dye DiI and injected i.p. to track their localization *in vivo*. Significant numbers of labeled cells were recovered from the airways (with peak recovery at 2 week post-treatment), but modest numbers of cells were also found within the lung-draining (mediastinal) lymph nodes (Appendix-1). The ingestion of FITC-coupled molecules via mannose receptor-directed macropinocytosis

has been described as an attribute of immature DCs (Sallusto, Cella *et al.* 1995). As expected, our putatively tolerogenic DCs were highly phagocytotic and this activity was downregulated in the mature DC_{TNF} population (Sallusto and Lanzavecchia 1994; Sallusto, Cella *et al.* 1995). The secretion of low levels of inflammatory cytokines IL-1 β , IL-6 and IL-12 by our DC_{IL-10}, in conjunction with their increased secretion of IL-10, further suggested their tolerogenic nature.

AHR, or hyper-irritability of bronchial smooth muscle is the hallmark of asthma (King, Pare *et al.* 1999) and so prevention of this response is an ideal outcome in asthma therapeutics. Thus, one of the most significant findings of this thesis was the abrogation of AHR in the severely allergic mice (i.e. showing more than 60% eosinophilia). AHR started to decrease within 2 weeks of treatment and was completely abolished by 3 weeks, indicating that smooth muscle-dependent bronchoconstriction was relieved and the lumen of the airways was fully patent. Our long-term assessments of the DC_{IL-10} treated animals showed that this therapeutic effect was maintained only until week \approx 10 post-treatment, and that a return of full AHR had occurred by 13 weeks. We are not aware of any reports of transient correction of AHR to date and at this time we have no experimental insights into the mechanisms mediating this effect, particularly when allergen-driven pulmonary Th2 responses remain tolerized well beyond this 3-month window.

Evidence suggests that AHR can arise via two distinct cellular processes. One is via mast cell activation (Busse 1998; Thomas 2001), while the other is IgE/mast cell-independent, but eosinophil/IL-5-dependent (Kobayashi, Miura *et al.* 2000). It has also been suggested that development of AHR may be a two-step process. The first step involves the IL-5/eotaxin-induced infiltration and activation of eosinophils in the airways, followed by release of sensory neuropeptides (e.g., neurokinin receptor-activating tachykinins) induced by eosinophil-derived

inflammatory mediators and causing epithelial damage and activation of sensory nerves (Kraneveld, Folkerts *et al.* 1997). Tachykinins, substance P, and particularly neurokinin-A (NKA) potently constrict human airway smooth muscles *in vitro* and have significantly greater potency in the smaller airways (Frossard and Barnes 1991). In guinea pigs it has been shown that AHR to histamine but not eosinophilia was completely blocked by pretreatment with neurokinin-2 receptor antagonist (Kraneveld, Nijkamp *et al.* 1997). In addition, in a mouse model of inflammatory bowel disease, it has been shown that IL-10 suppressed neurokinin-1 receptor (NK-1; binds substance P, a tachykinin) mRNA expression and played an important role in regulation of intestinal mucosal inflammation. Both substance P and its receptor NK-1 are found at increased levels in the lungs during airway inflammation (Kaltreider, Ichikawa *et al.* 1997). Therefore, there is a possibility that IL-10 secreted by our tolerogenic cells might directly inhibit neurokinin receptors and hence normalize AHR. The inflammatory responses could be downregulated by indirect effects of IL-10 on T cells or DCs as discussed below. We found that AHR returns at 13 weeks in spite of the continued dampening of the Th2 responses, but other experiments from our lab documented that re-treatment of post-13 week mice with another round of ^{OVA}DC_{IL-10} switches off the AHR again (Nayyar *et al.* manuscript in preparation). This is consistent with the direct therapeutic effects of IL-10 released by ^{OVA}DC_{IL-10} on AHR although other mechanisms may also be responsible.

Our lab has already reported that AHR develops in the absence of classical allergic disease (i.e. OVA-specific IgE, Th2 response and eosinophilia) following sensitization with limiting doses (i.e., ~100ng) of OVA-alum (Schneider, Li *et al.* 2001). In the present study, full tolerization of AHR occurred while IgE levels and eosinophil responses remained intact. Dampening of smooth muscle responses can be implemented even in the face of full spectrum

allergic responsiveness. It has been shown that expression of an IL-10 transgene within the airways suppresses AHR in the face of ongoing airway inflammation (Nakagome, Dohi *et al.* 2005). In addition, IL-10 can block smooth muscle cell activation by inflammatory stimuli *in vitro* or *in vivo*, at least in part by directly inhibiting I- κ B degradation and NF- κ B nuclear translocation (Mazighi, Pelle *et al.* 2004). Thus, IL-10 released locally by our $^{OVA}DC_{IL-10}$ could potentially have been directly responsible for amelioration of AHR, and very recent data from our lab that silencing of IL-10 expression within our $^{OVA}DC_{IL-10}$ eliminated their effects on AHR is consistent with this argument (Appendix-2). Furthermore, it has been shown previously that passive transfer into naïve mice of IL-10-expressing, but not IL-10^{-/-}, DCs from mice previously tolerized to aeroallergens can prevent the development of AHR (Akbari, DeKruyff *et al.* 2001).

One $^{OVA}DC_{IL-10}$ treatment led to a greater than 60% reduction in airway eosinophilia. The exposure of the mice to an OVA aerosol at the 3-week time-point led to a slight increase in eosinophilia, although it was significantly lower than that in the ALD, $^{OVA}DC_{TNF}$ and $^{OVA}DC_{GM-CSF}$ group animals. The overall eosinophilia was reduced with time, eventually to \approx 3% in the $^{OVA}DC_{IL-10}$ -treated animals (i.e., by 32 weeks; Figure 5.3.2), and repeated $^{OVA}DC_{IL-10}$ treatments reduced eosinophilia to less than 1% (Figure 5.4.2). The modest eosinophilia on allergen aerosol challenge (at 3 weeks) indicated that the host allergic reactivity had not yet been fully moved into a tolerance mode, but we venture that such high doses (i.e., 10 mg/ml) of antigen would not be encountered in a normal environment, so it could well be that lower doses of allergen would not have induced any eosinophil response. Similarly, the 30-60% decrease in OVA-specific IgE observed at 3 weeks, together with the greater reduction observed in the IgE response at 16 weeks, suggests that at 3 weeks tolerance was still progressing. That the repeated $^{OVA}DC_{IL-10}$ treatments reduced the OVA-specific IgE much more significantly at a much earlier time point

(Figure 5.4.3) suggests that achievement of tolerance is a progressive process that can be augmented by repeated treatments. A significant decrease in the IgG1 observed at 3 weeks was also maintained until 32 weeks, and this effect could similarly be augmented with repeated DC treatments. Interestingly, we also found that overall IgG1 and IgG2a levels started to increase from 19 week onwards and were dramatically increased at 32 weeks after aerosol challenge (Figure 5.3.3). This suggests that like AHR, Th2 tolerance also starts to wear off around this time with respect to antibody levels although significant differences still remained between $^{OVA}DC_{IL-10}$ and control groups. The levels of different antibody isotypes in the plasma further supports the argument that tolerance rather than Th1 skewing occurs, as the levels of IgG2a antibody (as well as IL-12, representative of Th1 responses) were similar in all groups, and more often than not were decreased compared to control groups at 32 weeks, as well as on repeated treatments.

Our $^{OVA}DC_{IL-10}$ treatments also affected the recipients' Th2 responses (i.e. BAL and parenchymal), but not via induction of Th2-to-Th1 immune deviation. IL-4 is a multifunctional cytokine that causes isotype switching of B cells to IgE synthesis (Bradding, Feather *et al.* 1992), IL-5 induces eosinophil expansion and recruitment (Ying, Humbert *et al.* 1997), and roles for IL-9 and IL-13 (Nicolaides, Holroyd *et al.* 1997; Wills-Karp, Luyimbazi *et al.* 1998) have been well documented in enhancement of eosinophilia and AHR. For example, IL-9 activates epithelial cells to secrete CCL11 (eotaxin) and other eosinophil chemotactic chemokines (Dong, Louahed *et al.* 1999) and also stimulates goblet cells to secrete mucus, and at least some of these effects are dependent on IL-13 co-expression (Steenwinckel, Louahed *et al.* 2007). The Th2 cytokine levels of BAL fluids from DC_{IL-10} -treated animals were markedly reduced compared to saline-treated ALD mice at 3 weeks and this trend was effectively maintained until 32 weeks, as well as following repeated $^{OVA}DC_{IL-10}$ treatments. On the other hand, the parenchymal responses were

moderately affected at the 3-week time-point but were significantly lower than those of saline-treated ALD control animals at 32 weeks or following repeated $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ treatments. Again, this suggests that ongoing tolerogenic processes gain dominance over time and that repeated treatments can augment this process. That Th1-skewing does not occur was also supported by the BAL and parenchymal levels of the Th1-response inducing cytokine, IL-12, which was either reduced or unaffected in the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated groups compared to the $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$, $\text{OVA}^{\text{DC}}_{\text{TNF}}$ and saline treatments.

Steady state DCs remain immature and can induce T cell anergy following interaction with naïve or memory T cells (Schwartz 2003). Furthermore, injection of immature DC in normal healthy humans can be tolerogenic (Dhodapkar, Steinman *et al.* 2001). The increased AHR and Th2 responses seen in our $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ -treated animals may be attributable to the fact that these cells gained access to a residual inflammatory environment (the airways), which would have activated them to become immunostimulatory. Expression of inflammatory cytokines in the airway inhibits tolerance induction by increasing local antigen presentation and thereby facilitating the development of immunoinflammatory responses to otherwise innocuous antigens (Stampfli, Wiley *et al.* 1998). Mature DCs show increased TNF expression (Chen, Gordon *et al.* 2002), while TNF-treated DCs mature both functionally and phenotypically and thereby effectively induce immune responses (Lutz, Kukutsch *et al.* 1999; Berthier-Vergnes, Bermond *et al.* 2005). We suggest that for these reasons both the $\text{OVA}^{\text{DC}}_{\text{GM-CSF}}$ and $\text{OVA}^{\text{DC}}_{\text{TNF}}$ treatment groups had increased AHR and Th2 responses compared to the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated group. These differences between the three DC groups were not evident at 3 weeks but became clearly obvious at 32 weeks. In addition, we found that IL-5 was the only cytokine that was significantly reduced in the $\text{OVA}^{\text{DC}}_{\text{IL-10}}$ -treated group compared to other DC controls at 3 weeks and neutralization

with IL-10R Ab also reversed BAL IL-5 levels at this time point. This suggests that both the $^{OVA}DC_{GM-CSF}$ and $^{OVA}DC_{TNF}$ showed transient reduction in Th2 responses early on and, like $^{OVA}DC_{IL-10}$ (which induce effective tolerance at 32 wks), induced immunostimulatory responses at later time points. IL-5 is probably the first Th2 cytokine to be effected in this series of tolerogenic events.

Interestingly, we found that with repeated $^{OVA}DC_{IL-10}$ treatments, the BAL levels of TGF- β , but not of IL-10, were substantially increased (Figure 5.4.4). CD4⁺CD25⁺ cells from IL-10^{-/-} mice that have been supplemented with recombinant IL-10 have been associated with elevated BAL levels of TGF- β and suppressed AHR and inflammation (Joetham, Takeda *et al.* 2007). It is possible that the Th2 responses might have been affected by IL-10 induction of TGF- β , and particularly as TGF- β -treated tolerogenic DCs have shown to reduce airway inflammation (Zhang-Hoover, Finn *et al.* 2005).

It has also been more recently shown in our lab that $^{OVA}DC_{IL-10}$ treatments have allergen-specific effects. In two of two experiments, treatment of OVA asthmatic mice with house dust mite allergen-loaded DC_{IL-10} ($^{HDM}DC_{IL-10}$) did not normalize the AHR at 1, 2, or 3 wk post-transfer (Appendix-3), although these cells were fully capable of tolerizing the AHR of HDM-allergic animals (Lu, *et al.*, manuscript in preparation).). These irrelevant allergen-presenting tolerogenic dendritic cells would nevertheless have been secreting IL-10, which suggests that the transient effect of $^{OVA}DC_{IL-10}$ on AHR would not be due to direct effect of this IL-10, but rather would necessarily involve other mechanisms. In those experiments, the airway eosinophilia of the mice treated 3 wk previously with $^{OVA}DC_{IL-10}$ or $^{HDM}DC_{IL-10}$, were reduced by 59.8 +/- 3.6% (p<0.001, versus $^{OVA}DC_{GM-CSF}$ mice; Appendix-3) or 4.2 +/- 4.4% (p>0.05, versus asthma-phenotype mice), respectively. The circulating levels of IgE and IgG1 were also significantly

reduced in the $^{OVA}DC_{IL-10}$ (Appendix-3), but not in the $^{OVA}DC_{GM-CSF}$ or $^{HDM}DC_{IL-10}$ -treated mice (for both, $p>0.05$ versus asthma-phenotype mice). In addition, others have reported antigen-specific effects of IL-10-modulated DCs (Henry, Desmet et al. 2008).

Lastly, on assessing the mechanisms involved in $^{OVA}DC_{IL-10}$ -induced tolerance we found that $^{OVA}DC_{IL-10}$, but not $^{OVA}DC_{GM-CSF}$ or $^{OVA}DC_{TNF}$, induced unresponsiveness in a memory T cell population (Figure 5.5.1.1). It has been shown that preculture of DC with IL-10 can induce a state of alloantigen-specific anergy in $CD4^+$ T cells and of peptide-specific anergy in the influenza hemagglutinin-specific T cell clone HA1.7 (Steinbrink, Wolfl *et al.* 1997). Furthermore, administration of recombinant IL-2 has been reported to reverse anergy (Schwartz 2003). Herein, our data show that IL-10 can induce a tolerogenic phenotype in DCs differentiated *ex vivo*, and that addition of IL-2 in our system partially reverses Th2 responses, as IL-13 was significantly increased (Figure 5.5.2.1). A partial reversal is consistent with tolerogenic process or at least in the induction of anergy (Itoh, Takahashi *et al.* 1999).

On assessing the role of different mediators which we thought might be involved (e.g., IL-10, TGF- β and IDO) we observed contrasting results *in vitro* and *in vivo*. No discernible effect was seen with the neutralizing anti-TGF- β antibody either *in vitro* or *in vivo*. On the other hand, although anti-IL-10R antibody did not affect *in vitro* responses, *in vivo* experiments showed that anti-IL-10R antibody given at a time anticipated to target the effector phase of tolerance induction significantly reduces eosinophilia and BAL IL-5 and IL-13 levels. Recently, to address a role for IL-10 expression by $^{OVA}DC_{IL-10}$ in tolerance more directly, Dr. H. Huang in our lab used an IL-10-specific siRNA approach and assessed the ability of the IL-10-targeting, versus nonsense control, siRNA-treated $^{OVA}DC_{IL-10}$ to induce tolerance. When he used these cells to treat asthma-phenotype mice, he found that the non-sense control siRNA-treated

^{OVA}DC_{IL-10} cells induced tolerance, while the IL-10 siRNA-treated cells had no discernible impact on AHR, pulmonary Th2 cytokine levels, airway eosinophilia, or circulating OVA-specific IgE or IgG1 levels (Appendix-2). This data indicates that IL-10 expression by ^{OVA}DC_{IL-10} is critical for the tolerogenic functions of these cells. There could be two possible explanations for why our neutralizing IL-10 was not highly effective. Either the levels of antibody employed were insufficient to completely neutralize IL-10, or timing of injection (day 12-21) may have been inappropriate. The doses of antibody used by others vary in the reported literature from 0.25 - 1 mg/kg (Castro, Neighbors *et al.* 2000; Liu, Hu *et al.* 2003; Hammad, de Heer *et al.* 2004). We used a dose that has been reported previously to effectively block IL-10 function and cause early death in relatively resistant mice with of trypanosomiasis (Shi, Pan *et al.* 2003). An inappropriate timing of delivery of the neutralizing Ab may also be another cause of its ineffectiveness, as we injected it starting on day 12 (i.e., when the physiological appearance of tolerance first occurred). As seen in Dr. Huang's experiment, when DCs that did not express IL-10 were delivered on day 0, there was no reversal of asthmatic responses observed. This implies that although effective physiological responses begin to appear at 2 weeks, DC_{IL-10} might interact within the lung environment ahead of time to initiate the events during first 12 days to set the ground for tolerance induction later.

Unlike our *in vitro* data, our *in vivo* data suggested a potential role in tolerance induction for IDO, a tryptophan-catabolizing enzyme that has regulatory effects on T cells. We found that 1-methyl tryptophan (1-MT) pellets on first use partially reversed Th2 responses (Figure 5.5.2.2B and C). Similar to the IL-10R and TGF- β neutralization experiments we started the 1-MT treatment on day 12. Both antibody responses and Th2 responses were enhanced compared to placebo-treated mice but were still significantly lower than ALD mice, again suggesting that

discernible tolerogenic events do take place during the first 12 days after DC_{IL-10} delivery. Recently, a report showed a probability of association between enhanced IDO activity and IL-10 production in clinically unresponsive aeroallergen-sensitized atopic subjects. This effect involved T cell tolerance mediated by APCs (von Bubnoff, Fimmers *et al.* 2004). We think that in our model system IL-10 might possibly be involved in enhancing IDO upregulation in DCs. The lung cells of tolerogenic mice showed mRNA levels expressed at levels two-fold greater than those of ALD mice (Figure 5.5.2.2D). It has also been shown that Treg can induce IDO expression in DC via a CTLA-4 dependent pathway (Fallarino, Frohman *et al.* 2003), which suggests that IDO's effects could also come into play after the induction of Tregs. Thus, IDO may have a role in this tolerance loop and this will require further assessment. We believe that these tolerogenic DCs interact with T cells directly to render them tolerogenic, and these now tolerogenic T cells in turn interact directly with other T effector cells (Zheng, Wang *et al.* 2004) or with DCs to induce them tolerogenic also (Frasca, Carmichael *et al.* 1997; Waldmann and Cobbold 1998) in an infectious manner (i.e., infectious tolerance).

It has been well acknowledged that interaction of T cells with DCs is important for generating appropriate immune responses to foreign antigen (Lanzavecchia and Sallusto 2001). The immunosuppressive effects of IL-10 on T cells are believed to be largely mediated indirectly by the effects of this cytokine on DC (Beissert, Hosoi *et al.* 1995; De Smedt, Van Mechelen *et al.* 1997). Moreover, we herein confirmed that IL-10-treated and -secreting DCs had therapeutic effects in allergic lung disease. Also, it has been reported that *in vivo* delivery of an IL-10 transgene suppresses airway eosinophilia and airway hyperreactivity even during the effector phase of airway inflammation (Nakagome, Dohi *et al.* 2005), and also that pulmonary DCs, which transiently produced IL-10, from mice exposed to respiratory antigen stimulated

development of Tr-1 like cells (Akbar, DeKruyff *et al.* 2001). We think that induction of regulatory T cells seems to be the most logical explanation of the therapeutic efficacy of IL-10-treated DCs although other possible mechanisms such as induction of regulatory B cells is certainly an alternate pathway that should be kept in mind. It has been shown that B cells, by producing cytokines such as IL-10, can act as regulatory cells in chronic inflammatory environmental settings (Mizoguchi, Mizoguchi *et al.* 2002)

In conclusion, IL-10 treatment of DC *in vitro* changes them to a tolerogenic phenotype. These cells can transiently induce complete reversal of AHR in our mouse model of severe allergic lung disease. These cells secrete modest amounts of IL-10, which H. Huang's data suggest is critical for inducing allergen-specific tolerance. Further, we think that AHR and Th2 reactivity might be differentially regulated. Normalization of AHR may be a direct effect of IL-10 on the smooth muscles or airway nerve receptors, while Th2 responses may be dampened by upregulation of Treg cells and/or rendering other DCs tolerogenic. DCs may either become tolerogenic by direct effects of IL-10 on other DCs (by downregulating the costimulatory machinery) or through upregulation of the tryptophan-catabolizing enzyme IDO (which mediates T cell suppression /regulation). Subsequent data (unpublished) generated in our lab by Dr. Hui Huang indicates that $^{OVA}DC_{IL-10}$ converts memory cells into regulatory cells both *in vitro* and *in vivo*. We thus think that our tolerogenic DCs possibly induce regulatory T cells that may then interact directly with other T cells in their vicinity to render them tolerogenic. This can occur by inducing of CD25⁻ T cells to differentiate into CD4⁺CD25⁺ Treg cells (Zheng, Wang et al. 2004), which can themselves induce CD25⁻ cells to differentiate to a CD4⁺CD25⁺ Tr1 regulatory cell type, establishing a cycle of tolerance. In addition, tolerogenic DCs or the regulatory T cells so induced may further interact with other DCs in the local environment to render them tolerogenic

by inhibiting their costimulatory signals, further inhibiting their antigen presenting ability, and thereby sustain infectious tolerance. Evidence suggests that, when injected into the lungs, CD8 α^+ DCs carrying immunogenic material can induce an immune response in the draining lymph node (Hammad, de Vries *et al.* 2004), and that allergen-presenting CD8 α^+ DCs can induce asthma tolerance (Gordon, Li *et al.* 2005).

To our knowledge, this is one of the few studies in established allergic lung disease model with positive therapeutic effects. The few studies done so far have either been undertaken when the therapy is given before or during the sensitization phase or before the aerosol challenge (Zhang-Hoover, Finn *et al.* 2005; Bellinghausen, Sudowe *et al.* 2006; Koya, Matsuda *et al.* 2007). Bellinghausen *et al.* show that IL-10-treated DC inhibit the Th1 and Th2 stimulatory activity of DC and don't affect systemic IgE and local airway inflammation, but do increase the IgG1 and IgG2a responses (Bellinghausen, Sudowe *et al.* 2006). This variability in the responses might result from varying doses or routes of DC delivery, time of delivery, and/or differences in the sensitization protocols. Bellinghausen *et al.* used 5×10^5 DCs delivered three times via the intravenous route, in contrast to ours (i.e. 10^6 DCs via i.p. or i.t. route). Interestingly we also found that our $^{OVA}DC_{IL-10}$ also had no effect when given i.v. (unpublished observation). We think the intratracheal as well as intraperitoneal route provides better therapeutic approach to the target organ i.e. the lung, which may explain the decreased eosinophilia, OVA-specific IgE as well as Th2 responses. In contrast, Koya *et al.* found that i.v. delivery of IL-10-treated DCs reduces AHR and airway inflammation in C57BL/6 mice (Koya, Kodama *et al.* 2006). More recently, Dr. M. Lu in our lab has shown that asthmatic C57BL/6 mice that are given 1×10^6 $^{OVA}DC_{IL-10}$ i.p. also become fully allergen-tolerant (Lu *et al.*, unpublished). One of the reasons for these discrepancies may be the difference in the DC populations used. The DC populations used by Koya *et al.* were

generated in GM-CSF and IL-4, and IL-10 was added at the starting of culture, generating comparatively immature DCs with low CD80, CD86 and MHC-II. In contrast, our DCs seem more mature, as IL-10 was added towards the end of culture. These DCs displayed with no differences in CD80 and CD86, but lower CD40 and CD54 expression relative to the mature DCs. The differences in the two reports might also be related to the route of DC delivery. Our results correlate well with those of Bellinghausen *et al*, (who used the BALB/c model) in which DCs given by the i.v. route were ineffective in inducing tolerance. Koya *et al* found no change in CD4⁺CD25⁺ T cells in OVA-pulsed versus -unpulsed IL-10-treated DC groups (Koya, Matsuda *et al*. 2007), which suggests that the effects induced by the IL-10-treated DCs were due to IL-10 secreted by DCs and not Treg cells. Thus, it is possible that either very few cells are able to reach the lungs (Lappin, Weiss *et al*. 1999) or IL-10 inhibits the migration of DCs from the blood vessels to the target site. Our DCs are delivered i.t., and so could potentially access the lung inflammatory cells, especially the tissue and the BALT lymphocytes, to induce potent therapeutic effects, potentially inducing induction of regulatory cells. Data generated subsequent to the completion of the work in this thesis indicates that ^{OVA}DC_{IL-10} cells given i.p. (Appendix-1) are able to reach the lungs and that they were as effective as DC given i.t. in terms of inducing tolerance.

To summarize, within 3 weeks of delivery into “asthmatic” mice, tolerogenic IL-10-treated DCs begin to ameliorate eosinophilia, and reduce OVA-specific IgE and IgG1, and BAL and lung parenchymal Th2 cytokine secretion. At this time AHR has been abrogated, but this effect is only transient. This Th2 tolerance is presumed to be infectious in nature as even after 8 months the IgE, eosinophilia, and Th2 responses remained relatively dampened. It is unlikely that our treatment DCs would have survived and secreted IL-10 for 8 months. Based on the

work in this thesis we can speculate that the tolerogenic effects initiated by $^{OVA}DC_{IL-10}$ cells would more than likely be maintained by other cells (regulatory DCs or T cells) which would keep active tolerance in place for prolonged periods. Subsequent work from our lab unequivocally confirms that both regulatory DCs and regulatory T cells are induced in DC_{IL-10} -treated asthmatic mice. Tolerogenic DCs have been shown by others to activate naturally-occurring regulatory T cells (nTreg) (Vigouroux, Yvon *et al.* 2004)), or to induce the differentiation of Tr1 (Battaglia, Gregori *et al.* 2006) regulatory cells, and plasmacytoid DCs are thought to influence the activity of myeloid DCs, at least under some conditions (de Heer, Hammad *et al.* 2004). We do not have evidence indicating which of these possibilities was operative in our induction of tolerance to allergic asthma, but the prolonged time course of the tolerance observed in our system suggests the establishment of a durable form of regulatory activity (Figure 6.1), such as might be associated with regulatory T cells (proposed model below, Figure 6.2).

Thus, IL-10-treated DC-induced tolerance would provide a novel approach to asthma therapeutics, but at the same time it poses challenges that need to be resolved. Primarily, we have to see if this therapy can be translated across to other strains of mice and eventually to humans. Two commonly used mouse models are C57BL/6 and BALB/c. If similar sensitization protocols are followed BALB/c are relatively high responders than C57BL/6 mice (Sugita, Kuribayashi *et al.* 2003) but both display similar characteristics of asthma i.e. AHR, airway inflammation and Th2 responses which could be ameliorated by $^{OVA}DC_{IL-10}$ therapy. Recently, Dr. M. Lu has completely characterized the asthma C57BL/6 model and confirmed the effectiveness of $^{OVA}DC_{IL-10}$ therapy in these mice. Furthermore, our *ex vivo* human experiments showed

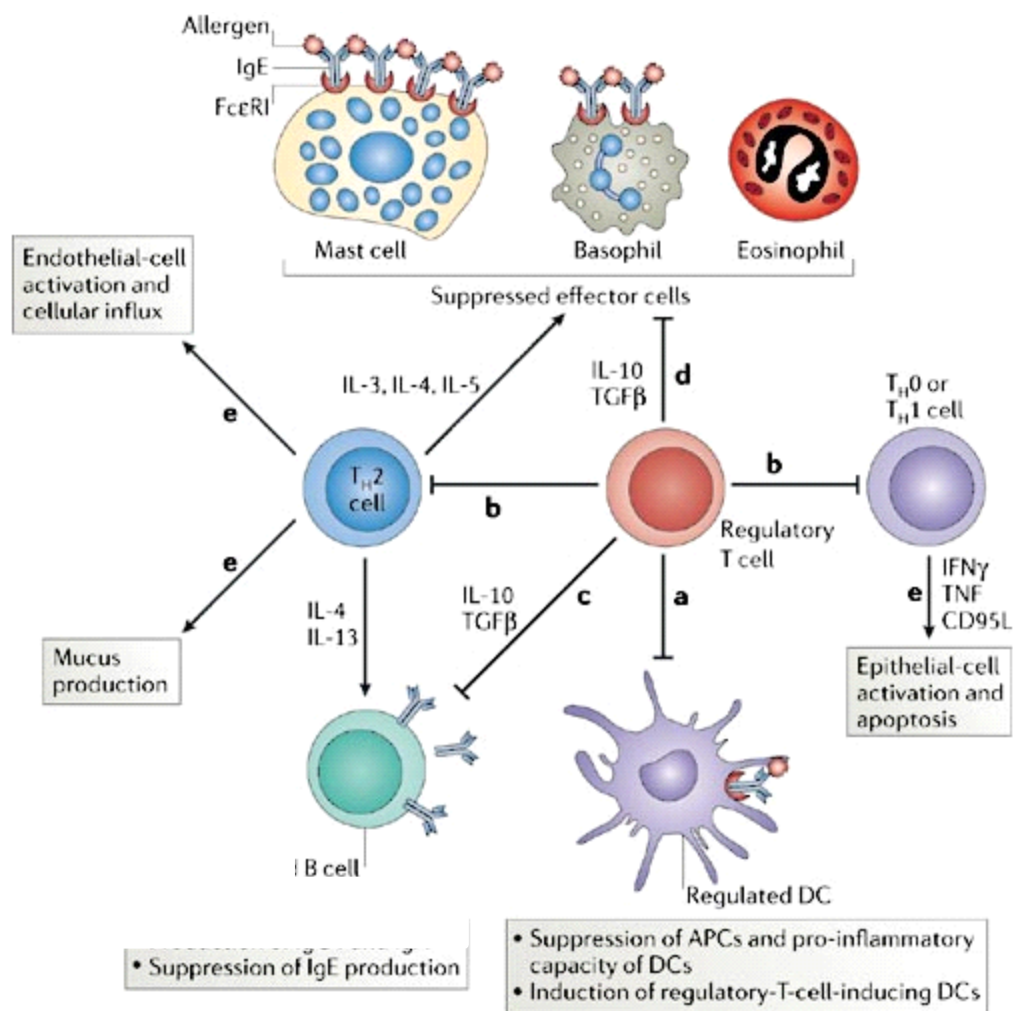


Figure 6.1 Effects of regulatory T cells and regulatory cytokines —IL-10 and TGF-β might contribute to the control of allergen-induced immune responses in five main ways: suppression of antigen-presenting cells (APCs) that support the generation of Th2 cells and Th1 cells (a); direct suppression of Th2 cells and Th1 cells (b); suppression of allergen-specific IgE production, (c); and suppression of mast cells, basophils and eosinophils (d). In addition, indirect inhibition of Th2-cell-associated phenomena (such as mucus production, and endothelial-cell activation and cellular influx) and Th1-cell-associated phenomena (such as epithelial-cell activation and apoptosis) is observed (e). (borrowed and modified from (Larche, Akdis *et al.* 2006)

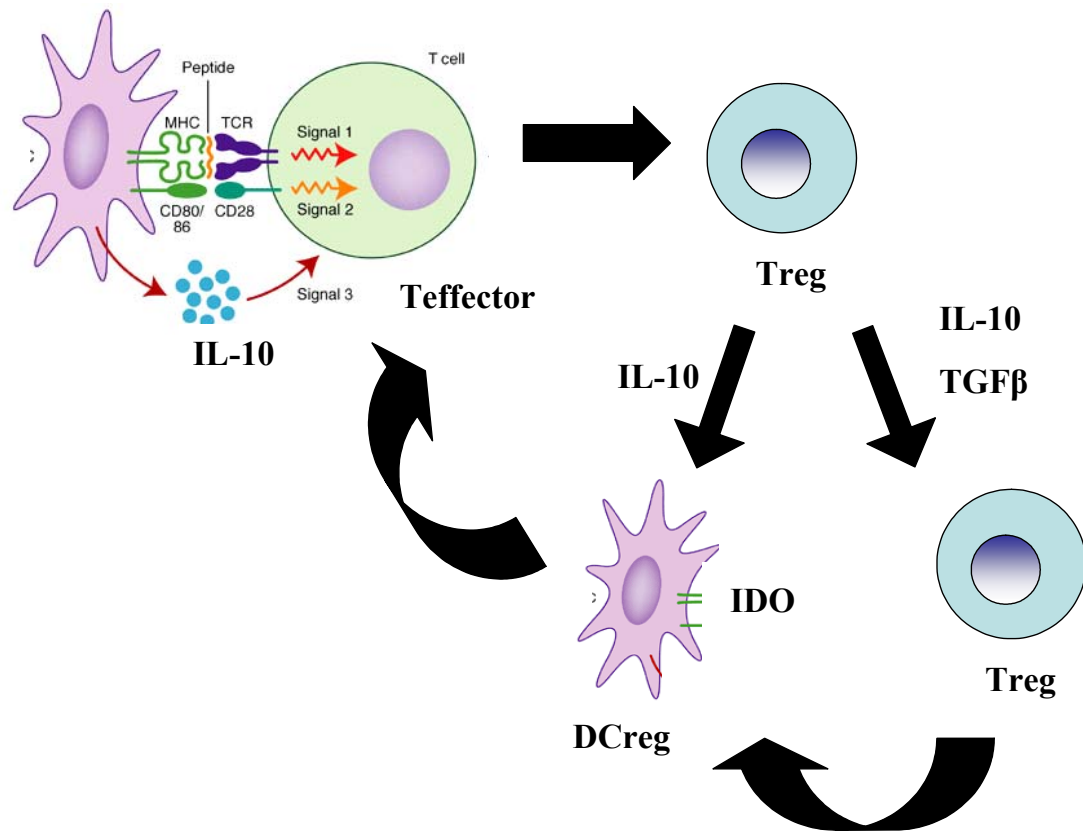


Figure 6.2 Hypothesized model of infectious tolerance induced by tolerogenic dendritic cells. When injected into the airway $^{OVA}DC_{IL-10}$ interact with the effector memory T cells and possibly converts them into antigen-specific regulatory T cells. These Tregs may secrete immunoregulatory cytokines like IL-10 or TGF- β , which would further induce Tregs in the lung environment. In addition, these Tregs could also induce regulatory DCs, possibly via IDO upregulation. IL-10 released endogenously or by Tregs may also induce regulatory DCs that can further induce Tregs thus maintaining the cycle of infectious tolerance for prolonged periods.

induction of Th2 tolerance by DC_{IL-10} loaded with the allergens (e.g., HDM, grass, cat dander etc.) to which the subject was sensitive. Secondly, we induced antigen-specific tolerance to OVA but human subjects experience multiple allergies. Thus, suppressing immune responses to one antigen may not affect responses to others and this would be a challenge to clinical implementation of immunotherapy. Perhaps, the subject could be treated first for the allergen to which he/she is most sensitive and then later for lesser allergens. This would require repeated treatments but likely substantially be less often than required for SIT. Whether DC loaded with different allergens can be mixed and given at the same time to multi-allergic subjects is another question that remains to be answered.

Thus, this amelioration of allergic responses by ^{OVA}DC_{IL-10} therapy in asthmatic mice may well provide valuable insights into its successful implementation in asthmatic humans. Although a mouse model does not reproduce all the features of human asthma, mice develop a clinical syndrome that in many aspects closely resembles human asthma. We venture that our model shares more in common with human asthma than other models reported so far. Major studies have used primary sensitization and challenge models (not the case in humans). In our model, initial sensitization and allergen challenge was followed by a recovery period and then a single provocation challenge was given, mimicking the situation encountered in asthmatic subjects. Further studies involving use of this therapy in humanized mice or other models closely related to humans (e.g., primates) may provide valuable information in determining its therapeutic effects. Although, it's still very far from being applicable for treatment of human asthma, IL-10-treated DC therapy still provides information that has made us a step closer to achieving the goal. Thus, with limited availability of data, my hypothesis is still acceptable i.e.

allergen-presenting interleukin-10-treated bone marrow-derived DCs can be used for therapeutic induction of tolerance to severe allergic lung disease in a mouse model of asthma.

CONCLUSIONS

1. The IL-10-treated DCs were avidly phagocytic, expressed high levels of IL-10, responded strongly to CCR5 ligands in chemotaxis assays, and expressed low levels of cell surface markers CD40 and ICAM-1.
2. Within 3 weeks of delivery into “asthmatic” mice, tolerogenic IL-10-treated DCs begin to ameliorate eosinophilia, and reduce OVA-specific IgE and IgG1, BAL and lung parenchymal Th2 cytokine secretion, and AHR was completely abrogated (but only transiently affected).
3. The induction of AHR and Th2 tolerance suggests that these two parameters are controlled independently in this model system.
4. This tolerance appears to be infectious in nature, as even after 8 weeks IgE, eosinophilia, and Th2 responses were relatively dampened and maintained for 8 months.
5. Repeated ^{OVA}DC_{IL-10} treatment kept AHR normalized and augmented the reduction in eosinophilia, OVA-specific IgE and IgG1 as well as BAL and parenchymal TH2 responses.
6. Both the i.t. and i.p., but not s.c., routes of delivering tolerogenic cells were effective in ameliorating asthmatic responses.
7. Interleukin-10 and possibly IDO seem to contribute to tolerogenic DC-mediated effects.

Appendix -1*

Table I. Recoveries of DiI^{hi} OVA⁺ DC_{IL-10} from tissues of recipient asthma-phenotype mice at various times after i.p. delivery.

TISSUE	Percent DiI ^{hi} cells ^a among the tissues assessed					
	4 dy		14 dy		21 dy	
	Saline	DiI-DC	Saline	DiI-DC	Saline	DiI-DC
Peritoneal cavity	0.53	24.9	0.08	64.1	<0.01	77.6
Mesenteric LN	<0.01	0.05	0.44	0.63	0.08	0.34
Spleen	<0.01	0.36	0.28	0.06	<0.01	0.01
Airway	0.04	2.56	0.37	15.6	0.15	2.25
Lung parenchyma	0.02	0.05	0.23	0.02	0.02	0.02
Mediastinal LN	<0.01	1.48	1.53	4.99	0.84	5.58

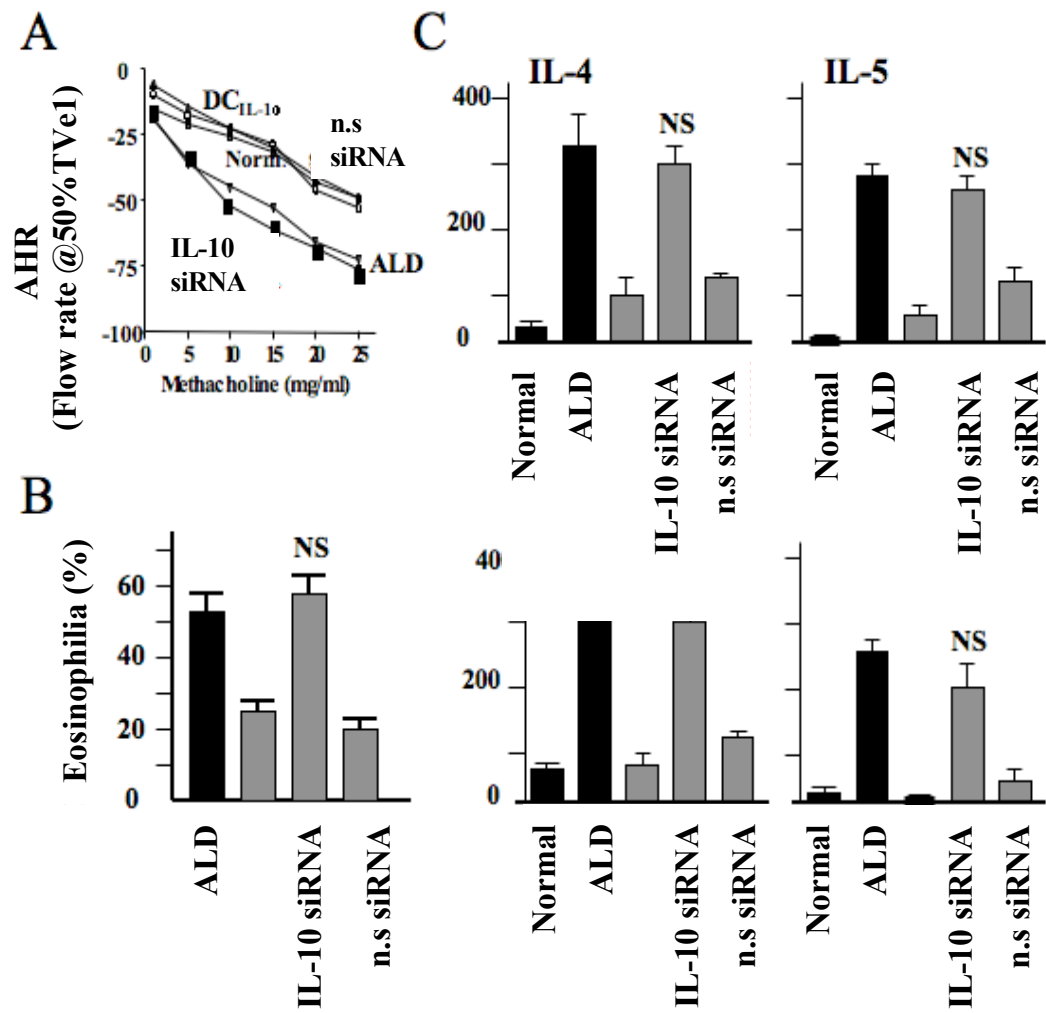
^a IL-10-differentiated DC were labeled with the lipid dye DiI, then injected i.p. into mice with asthma-like disease. At 4, 14, or 21 dy thereafter, cells were harvested from various tissues of the DC recipients and analysed by FACS for DiI-positive cells.

*Contributed by Dr. Meiping Lu

Appendix -2*

Silencing IL-10 expression within $^{OVA}DC_{IL-10}$ ablates their abilities to induce tolerance in mice with allergic lung disease/asthma. ALD was induced in mice as in Figure 5.2.1, then the animals were given otherwise normal $^{OVA}DC_{IL-10}$ (-) or $^{OVA}DC_{IL-10}$ that had been transfected with either scrambled (n.s. siRNA) or IL-10-specific siRNA. The IL-10 siRNA $^{OVA}DC_{IL-10}$ expressed normal levels of cell surface antigen-presenting machinery and CCR5, but did not express detectable levels of IL-10 protein or mRNA, or CCR7. The mice were otherwise treated as the animals in Figure 5.2.1 and euthanized at wk 3. **(A)** $^{OVA}DC_{IL-10}$ that had been transfected with scrambled siRNA induced ALD tolerance as well as otherwise untreated $^{OVA}DC_{IL-10}$, while $^{OVA}DC_{IL-10}$ that had been transfected with IL-10-specific siRNA were incompetent at reversing AHR in the recipients. Similarly, the silencing of IL-10 expression in the $^{OVA}DC_{IL-10}$ eliminated tolerance induction with respect to **(B)** eosinophilic inflammation and **(C)** BAL IL-4, IL-5, IL-9, and IL-13 responses to allergen provocation in recipients. The scrambled siRNA-treated cells were no different than untreated $^{OVA}DC_{IL-10}$ in inducing tolerance. NS, $p>0.05$ versus ALD mice.

*Contributed by Dr. Hui Huang



Appendix -3*

DC_{IL-10} induces allergen-specific tolerance in asthma-phenotype mice. An asthma-like disease (ALD) was induced in BALB/c mice as indicated earlier. Two weeks later, the animals were given saline (ALD), or 1×10^6 OVA-pulsed immature DC (Imm. DC) or DC_{IL-10} (^{OVA}DC_{IL-10}), or house dust mite allergen-pulsed DC_{IL-10} (^{HDM}DC_{IL-10}) by intratracheal injection. **(A)** Over the next 3 wk the AHR of the animals was assessed by head-out whole body plethysmography, in which the animals were exposed to increasing doses of Mch and their pulmonary airflow was measured as running 1-sec means at the 50% point in the expiratory cycle (Flow@50%TVe1) using a linked pressure transducer. From 14-21 day after cell transfer, the ^{OVA}DC_{IL-10} recipients normalized their bronchial responsiveness to Mch, while the ALD and Imm. DC-treated animals remained hyper responsive. ALD mice treated with ^{HDM}DC_{IL-10} did not correct their AHR. **(B)** On day 21, following the assessments of AHR, the animals were exposed to nebulized 1% OVA aerosols for 20 min, and then 48 hrs later they were sacrificed. We performed bronchoalveolar lavages (BAL) on each animal and quantified their airway eosinophilia from differential cell counts and BAL cytokine levels by ELISA. We also measured the OVA-specific plasma IgE and IgG1 antibodies by ELISA. The ^{OVA}DC_{IL-10} treatments had reduced the airway eosinophilic inflammation at 3 wk after cell transfer, as well as the circulating levels of OVA-specific IgE and IgG1. The ^{HDM}DC_{IL-10} did not reduce either of these parameters

* taken from Nayyar *et al* in preparation

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